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# KINETICS OF AEROBIC UTILIZATION OF MIXED SUGARS BY HETEROGENEOUS MICROBIAL POPULATIONS

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# KINETICS OF AEROBIC UTILIZATION OF MIXED SUGARS BY HETEROGENEOUS MICROBIAL POPULATIONS

By

SAMBHUNATH GHOSH

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## AUTHOR

SAMBHUNATH GHOSH was a candidate for the degree of Doctor of Philosophy in the School of Civil Engineering, Georgia Institute of Technology. His doctoral dissertation comprises the substance of this report.

# PREFACE

The influents to a system employed for the biological stabilization of organic wastewaters frequently contain multiple sources of carbon and energy necessary for microbial metabolism. Each of these sources of carbon and energy (hereafter called substrate) has the potential of controlling the microbial growth rate at one stage or another. It is generally assumed that a microbial culture assimilates all of the substrates simultaneously. However, the pioneering work of Monod with pure cultures revealed that the presence of a specific substrate constituent (such as glucose) caused competition between the substrates for controlling the rate of growth. Under these circumstances, the microorganisms preferentially assimilated one substrate at a time while temporarily preventing metabolism of the others. Therefore, the corresponding batch growth curves were characterized by sequential growth cycles with each cycle corresponding to the exclusive utilization of a specific substrate of the mixture in separate phases.

A few studies have been reported in the past ten years showing that phasic uptake of competing substrates can also be brought about by heterogeneous cultures employed in biological waste treatment processes. However, the conclusions of the investigators have been contradictory. Some have shown that glucose and galactose are sequentially removed by a heterogeneous batch culture whereas others have found these same substrates to be simultaneously assimilated in a batch process employing organisms having their origin in waste treatment plants.

Moreover, glucose and sorbitol have been shown to be assimilated simultaneously or in separate phases in batch cultures depending on the "age" of the culture. Some researchers have also investigated the fate of competing substrates in continuous biological processes. Continuous culture studies have shown that competing substrates can be simultaneously assimilated by heterogeneous microbial populations although these same substrates were utilized sequentially (i.e., in separate phases) during batch growth. Clearly, the results of continuous culture studies were both in agreement with and contradicted by the results of the batch culture studies. Unfortunately, few efforts have been directed toward resolution of these contradictions. The conditions under which competing substrates will be sequentially or concurrently assimilated have not been established. Furthermore, information regarding kinetics of growth and removal of the competing substrates is not available.

The causes and kinetics of sequential and/or concurrent utilization of competing substrates are of great importance for prediction of the performances as well as for the design and control of biological treatment processes. Since such information has not become clearly established, the objectives of this research were:

1. to investigate the role of the environmental, biochemical or other factors responsible for the occurrence of phasic or concurrent assimilation of two competing substrates;

2. to obtain basic information necessary for formulation of mathematical models describing the kinetics of assimilation of the competing substrates; and

3. to identify the probable cellular mechanisms that regulate the pattern (sequential or concurrent) of substrate assimilation.

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Permission has been granted by the Graduate Division for Mr. Ghosh's dissertation to be published as a report of the Water Resources Center, Georgia Institute of Technology.

## SUMMARY

The influents to biological wastewater treatment processes frequently contain several different carbon and energy sources, each of which could be growth limiting. These substrate components may interact because the enzyme systems capable of catabolizing each substrate compete for supplying precursors for biomass synthesis. The substrate capable of supplying the anabolic pathways with needed precursors at the fastest rate is the *primary substrate* and is preferably catabolized. In some systems the enzymes of the *secondary substrates* are so regulated that the uptake of these substrates can be initiated only after a period of preferential utilization of the primary substrate. This research was undertaken to identify some of the environmental, physiological, and biochemical factors responsible for bringing about substrate interactions. The study was designed to provide information pertaining to the kinetics of growth and assimilation of competing substrates.

The investigations were performed in a chemostat type reactor maintained at approximately 20°C. The influent contained glucose and galactose as the competing substrates along with other non-limiting nutrients. Three sets of experimental runs were carried out using galactose, glucose, and galactose-glucose mixtures, the latter in concentration ratios of 1.05 to 1.23:1. Comparison of substrate utilization rates and cell yields from each of the three substrate systems at selected growth rates provided qualitative as well as quantitative information pertaining to the nature of substrate interactions and the kinetics of uptake of the competing substrates.

The steady state data for all substrate systems showed that at high detention times (above 1.7 hours for glucose and 2.9 hours for galactose) slow growing microorganisms, characterized by a lower maximum specific growth rate, $k^m$, and a lower saturation constant, K, prevailed over the fast growers. At lower detention times the slow growers were selected against and microorganisms with a higher $k^m$ and K became dominant. Steady state concentrations could be described by the Monod expression for specific growth rate.

Glucose was found to be far more efficient than galactose in terms of the growth yield and specific growth rate attained at any substrate concentration. These two substrates were simultaneously assimilated from influents containing mixtures of the two. Glucose consumption, which remained unaffected in the presence of galactose, accounted for the growth yield, while assimilated galactose accounted for the energy of maintenance of all cells. With a mixed substrate, glucose controlled the growth rate, as this substrate sustains a higher cell yield and a faster rate of reproduction. Glucose stimulated the utilization of galactose as a supplementary energy source even though the galactose uptake rate per unit biomass was reduced considerably through noncompetitive and feedback-type inhibition of the rate controlling galactose permease.

Theoretical analysis indicated that in batch or plug flow reactors the slow growers would sequentially assimilate glucose and galactose at growth rates below 0.6/hour. However, the fast growers preferred concurrent utilization if the ratio of initial galactose and

glucose concentrations exceeded 1.25:1. Sequential utilization was not evidenced in the continuous flow system as the slow growers were replaced by organisms capable of concurrent assimilation. Thus, the mode and kinetics of assimilation (sequential or concurrent) of competing substrates are determined by the nature of the specific growth rate functions with reference to the individual substrate concentrations, the relative configurations of the resultant specific growth rate curves of the various substrates, the ratio of concentrations of the substrates, and the type of flow model used for the culture system.

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# CHAPTER I

# INTRODUCTION

## Biological Cultures

### Microbial Fermentations

Man was well aware of the phenomenon of fermentation in ancient times, even though he did not know of the existence of microorganisms and their role in causing this phenomenon. Nevertheless, he keenly observed the phenomenon and learned to put it to work for his benefit. The aging of meat for a more pleasing taste and the manufacture of intoxicating drinks from grains and fruits were man's first use of fermentation. The French scientist Thenard (1) announced in 1803 that the "essential materials" used by wine makers were living organisms responsible for producing the alcohols. However Thenard's discovery was not to be appreciated until 1857 when Pasteur (2,3) published his research showing that certain diseases were caused by living microorganisms and that faults in wine were due to improper alcoholic fermentations carried out by living cells known as yeasts. It is the latter property, of course, which is still the subject of much concern to biochemical and sanitary engineers. Since the days of Pasteur a vast body of knowledge has been accumulated about the structure, food and environmental requirements, growth patterns, and hereditory characteristics of microorganisms.

### Role of the Environment

The microbes form a large group of very diverse organisms --

diverse in their characteristic food habits and habitats -- and can be found almost anywhere they are sought because there almost always exist microenvironments suitable for the survival of one or more of the numerous varieties of microbes. Wind, water, and animals help in their migration and their making contact with the right habitat and food. Since the major constituent of a microorganism is water, ranging from 75-90 percent, the habitat should necessarily be aquatic or humid in nature. The chemicals which give rise to optimum growth are called nutrients and they are of two categories: (1) the dispensable nutrients which can be done without, and (2) the indispensable nutrients which have to be present in order for growth and multiplication to take place. Some of the nutrients like carbon, nitrogen, phosphorus, sulfur compounds, and most metals are incorporated into the protoplasm, while others such as oxygen, ammonia, hydrogen sulfide, glucose, etc., which might serve as electron acceptor or energy sources. are used and subsequently disposed of. Proliferation requires optimal levels in the environment of physical and chemical factors such as surface tension, osmotic pressure, temperature, pressure, hydrogen ion concentration, and salinity. The optimum generally consists of a fairly wide range of values, but different species* are not all alike in this respect. It is also fairly well known that radiation and the nature of solid surfaces in the environment affect growth, the effect of the latter being favorable and attributed by microbiologists to the concentration of nutrients on solid surfaces by absorption.

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*Every organism belongs to a species which is the lowest subdivision of the kingdom. The word strain designates a laboratory culture derived from a known source of some species. Strictly speaking, the basic unit of taxonomy is the strain or clone which is a population of genetically identical cells and would give rise to a strictly pure culture.

Mixed Cultures

An environment along with the organisms which are cultivated in it can be referred to as a biological culture. In nature, it is possible that more than one species of bacteria can find an environment suitable for growth and multiplication either free of interference from each other or by beneficial association. Such cultures, which may be termed mixed cultures, invariably change the environment physically and chemically in the process of growth and finally to the detriment of further multiplications of certain species. Other micro- or macroorganisms may find the changed environment suitable, and grow by consuming the remnants of food, waste products, and even the bodies of the first group of inhabitants. Change in the physical and chemical properties of the environment, whether brought about by the activities of the microorganisms or macroorganisms, would give rise to a succession of biological population in time. The occurrence of the culture of a particular species or a group of species in a particular locale in nature is frequently discontinuous in time since the conditions permitting the persistence of species by multiplication may appear and disappear in regular or irregular fashion. One can thus begin to appreciate the shifts of microbial population which may and indeed do occur due to the change in physical as well as chemical characteristics of the environment. It might be said that an environment "selects" one or more species of microorganisms whose environmental requirements are compatible with those provided by the environment doing the selection. This is the area of study of the ecologist, and the biochemical and sanitary engineers find useful application of his findings in manipulating the environment for selecting the desired species from a mixed culture obtained from nature.

Pure Cultures

For centuries microorganisms have been playing far more important roles than their sizes would suggest. They are responsible for weathering and soil formation, elemental transformations, production of organic matter, causation of disease, decomposition of organic remains, production of antibiotics, vitamins and growth factors for higher animals, biological nitrogen fixation and fertilization of soil and aiding in digesting processes of ruminants and higher animals. Because of the vital roles that they play in determining the fate of human society, considerable importance has been attached to studying the morphology, physiology, biochemistry, genetics, and the life cycle of the microorganisms; these are conveniently accomplished by isolating and examining genetically similar types of organisms. A culture of microbes arising from parents which were genetically similar is referred to as a pure culture. Investigations with pure cultures of the appropriate organisms have led not only to control of disease and increase in life expectancy of humans and animals, but also to increase in production from age-old fermentation processes and introduction of new ones. Suffice it to mention that without pure culture studies such drugs as penicillin would not have been produced.

Were it not for the introduction of the elective or enrichment culture technique of Beijerinck (4) and Winogradsky (5), attempts to develop pure cultures might not have been very productive. In the enrichment technique, the microbiologist uses the knowledge of the ecologist for simulating the favorite natural microenvironment in the laboratory in macroscopic dimensions. The simulation technique is most useful for selecting organisms of special physiological groups, such as nitrifying, denitrifying, sulfur-oxidizing, and cellulose or protein-decomposing bacteria. Depending on the extent of knowledge about the nutritional properties and optimum levels of physical factors needed for growth, the adjustment of the environment in enrichment culture may be made so precise as to select and allow the growth and proliferation of microorganisms belonging to the same species. It is not difficult to imagine now that by graduated manipulation of the environment, Nature or man can obtain biological cultures exhibiting a spectrum of taxonomic heterogeneity ranging from highly heterogeneous to genetically homogeneous or pure culture. Though a common criticism of the use of pure cultures is that the organisms so obtained are "abnormal" and their physiological capabilities differ greatly from organisms in nature, yet one finds algal blooms in certain lakes to be restricted to a single species and single species of sulfur bacteria in sulfur springs. There are many other examples of natural pure cultures to be found in the microbiological literature.

## Dominant Cultures

It is conceivable that a given environment can hardly be suitable for proliferation of an indefinitely large number of species, and it is the experience of the ecologist that most natural environments are dominated in population by relatively few species -- half a dozen or so species or strains constituting 95 percent of the population. The situation arises by virtue of the fact that usually a few selective enrichment factors like high temperature, extreme pH, a toxic factor, or the presence or absence of a key growth factor operate to impose a limit on the taxonomic heterogeneity of the microbial population. Although it is in vogue to refer to natural biological cultures as heterogeneous, very often what one is speaking of is a culture of defined heterogeneity or a dominant culture.*

A biochemical engineer employs a pure culture and less frequently a dominant culture as dictated by the product of interest. A biochemical engineer's concern is the product and its purity, and very often such objectives are best achieved by selection of an appropriate pure substrate** to be processed by a pure culture. In contrast, a sanitary engineer has no choice over the substrate, which is almost always highly heterogeneous in chemical composition, and is responsible for producing innocuous products within acceptable limits. Dominant cultures are of best service due to the heterogeneous nature of the substrate. By properly manipulating the chemical and physical environmental factors, it is possible to maintain a dominant culture for yielding products of desired quality. The sanitary engineer thus uses dominant cultures for controlling biotic or abiotic environments, natural or artificial, whether they occur in the hydrosphere, lithosphere, or the atmosphere.

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*Very often the words mixed, heterogeneous, enrichment, and dominant are used interchangeably.

**In the literature the words nutrient and substrate are frequently used synonymously, but in this report the growth-controlling carbon and/or energy source would be designated as the substrate.

## Biological Cultures in Sanitary Engineering

It is the task of the sanitary engineer to restore the ecosystem[*] to a state acceptable to society and to maintain prescribed standards of quality which tend to be altered as a result of deposition of waste materials. This has traditionally been accomplished by a two-pronged attack: (1) adjustment of the environment of the ecosystem itself; and (2) control of the inputs of extraneous waste materials into the natural ecosystem by isolating the wastes and subjecting them to appropriate treatment before release into the ecosystem. The essence of the task is to convert the discarded organic matters into innocuous inorganic matter and acceptable gaseous products such as $CH_4$, $CO_2$, $N_2$, and $O_2$. This would not seem to be a difficult task since, by virtue of higher entropy, organic matters are metastable and their decomposition into simpler inorganic compounds would be exergonic. However, a certain amount of energy of activation is required to raise organics to a state of chemical reactivity, which may be accomplished by supplying energy from the outside and which can be recovered at the end of the reaction. The initial supply of energy required can be lowered by the addition of catalysts which help lower the energy of activation. However, chemical conversion of dilute organic wastes is not usually economical owing to the large heat requirements in heating carriage waters.

In biological conversion of organic matter, as carried out inside a microbial cell, a series of coupled biochemical reactions take part in the conversion process, each reaction being mediated by a biological catalyst called an *enzyme* and requiring a small amount of energy of activation. The process proceeds at almost a constant temperature and only a part of the energy of reactions appears as heat in contrast to a drastic rise in temperature and unchecked evolution of heat in purely chemical reactions. The remainder of the energy of reaction is conserved as chemical energy in the *energy-rich* phostate bonds of *energy-rich* compounds such as adenosine triphosphate (ATP). These important aspects of the biochemical reactions have led the sanitary engineers to use biological decomposition for destruction of organics in dilute aqueous wastes. A successful exploitation of this controlled and stepwise execution of endergonic reactions by the microbes, developed through evolutionary processes for their survival, would mean skilled harboring of these living micro-reactors. An artificial ecosystem is created to simulate the natural ecosystem which allows the flourishing of the desired organisms. Physical and chemical factors are so controlled that the chosen organisms can grow and multiply at the highest attainable rate.

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[*] An ecosystem refers to all the interacting biotic and abiotic (physical and chemical) elements in a limited and defined universe.

One point which is yet to be discussed is the fate of the stored energy from biochemical reactions. The important function of microorganism is to utilize nutrients for synthesis of macromolecules to form part of the protoplasm. The energy for this synthetic process is derived from cleavage of energy-rich bonds of the energy-rich compounds. The end result is that only a fraction of the organics of wastes can be knowledge of a process will be unavailable at the time of need and no rational model or mathematical equation can be derived which will be universally applicable to the prediction of the overall process behavior for different wastes. The design of the process is then based on the engineering judgment of the engineer and past experiences in the profession regarding the correlation between performance and operational variables in similar processes. In many cases the engineer establishes the correlation through laboratory or pilot plant studies. This empirical approach in the absence of needed information has been common in all branches of engineering and sanitary engineering is no exception. Such efforts cannot be belittled as they have been responsible for important concepts and methods of sanitary engineering. It is desirable, however, to appreciate the limitations of the applicability of these empirical relationships.

Process economics demand a more rational or even a deterministic approach to process design. The goal is rather simple, namely, carrying out the biochemical reactions in the shortest possible time, using the smallest possible reactor, and involving a minimum of process control and use of equipment and power. The first two are interrelated and involve maximizing the rate of biological activity which in turn involves increased process control and cost. A balance may be found by sacrificing the process rate (kinetics) and gaining in process control or "operation" cost.

## Process Kinetics

### Deterministic Model

The rate of conversion of the organic matter in waste waters is necessarily determined by the velocity of the coupled biochemical reactions in a microbial cell, and it should be possible, at least theoretically, to derive expressions for conversion rates of each nutrient and formation rates of each product. Such a deterministic approach is not presently possible owing to the complicated nature of the reaction arrangements forming a network with open-ended branches. Reactants are channeled into different directions and products might emanate from different chain reactions. The reaction of one chain might depend on the supply of products from reactions of another chain. The picture is further complicated due to transport kinetics of the nutrients through the cell wall and of products of reactions from one area of protoplasm to another. Some of the transport processes are mediated by enzymes while others are not. The control of the formation of the enzymes and their activities or both by the genes in response to stimulation from the extracellular environment complicates the picture enormously. Many of the reaction mechanisms, enzyme regulation mechanisms, and transport mechanisms are unknown. Furthermore, the nature of the complexity differs from cell to cell owing to the physiological heterogeneity of the dominant culture employed. It cannot be overemphasized that building of a deterministic process kinetic model is a formidable, if not impossible, task. The complexity of the problem, however, has not deterred the engineer in his effort to find a rational process kinetic model.

### Rational Model

In biological processes employing dominant cultures, it is the collective biochemical capability of the population which is of importance. The individual species of the process culture, though different in their total physiological capabilities, are controlled to collectively express a property, such as conversion of starch and $NH_4Cl$ to proteins, $CO_2$ and $H_2O$. The culture therefore may be considered uniform and akin to pure culture as far as its functions in the process are concerned. It is not surprising to find, therefore, as is very often the case, that the course of nutrient consumptions, product formation, and biomass synthesis follows orderly forms of mathematical equations with functions applicable to description of similar properties of pure cultures. The similarity of behavior of dominant and pure cultures indicates that the various species of a dominant culture grow in harmony without impeding each other's growth and proliferation and at a rate commensurate with the controlled environmental factors. Despite the skepticism of many, the concept of dominant culture behavior and experimental observations thereof points to the applicability of pure culture kinetics to dominant culture behavior. A convenient approach has been to construct a process kinetic model similar in form to those applicable in pure cultures, but adequate to describe the total behavior of the dominant culture as expressed by the course of nutrient assimilation or growth.

The concept of constructing a process kinetic model based on the kinetics of assimilation of organic carbon -- expressed as biochemical oxygen equivalent (BOD) or chemical oxygen equivalent (COD) -- dates back to 1909 when Phelps (6) proposed a monomolecular rate of biochemical degradation of organic matter which must "be a complete food or be combined with other essential food requirements." Phelps essentially proposed first order kinetics -- which does not necessarily follow from the monomolecular reaction -- with respect to the decomposable carbonaceous matter. Though the equation found almost universal acceptance and application in the following decades, it has also been the subject of criticism by many since first order kinetics are not applicable in all phases of microbial metabolism. A modern approach, which is more rational -- at least in the opinion of this author -- is to relate the kinetics of decomposition of nutrients to the kinetics of growth of the dominant culture since the integral property and behavior of the culture are reflected in the overall growth of the culture. The kinetics of assimilation of nutrients or formation of product can then be related to the kinetics of growth through the introduction of a coefficient or constant of proportionality between yield of biomass per unit mass of nutrient assimilated or product formed. This approach, although free from the criticisms associated with the first order approach, also has some limitations as discussed in Chapter II. Various equations have been proposed to describe the course and kinetics of growth, but the one due to Monod (7) has proven to be applicable for growth of dominant and pure cultures of bacteria and other microorganisms. The Monod model is flexible in that it can be used for zero-order kinetics of growth in the presence of abundant food supply up to second order kinetics when growth is controlled by limitation of a nutrient.

Up to this point the discussion has proceeded with the tacit assumption that the medium of growth is a "complete food," i.e., all required growth factors are present in quantities exceeding the minimum requirement, and as the organisms grow, one of the nutrients (substrate) is exhausted to the point of controlling further growth. The medium is in essence similar to the minimal media used in laboratory studies where not more than one chemical is provided to serve as the source of one (or more) growth factors. For example, a sugar may be used to serve as a source of carbon as well as energy.

## Substrate Interaction and Diauxie

If the minimal requirement is not satisfied, growth cannot occur and the addition of the deficient nutrients, such as nitrogen and phosphorus compounds, may be warranted. On the other hand, situations are found -- which are probably more frequent in waste treatment than realized -- in which there may be a multiplicity of sources of any of the growth factors. Thus, two or more sugars may serve as the source of cellular carbon; $NH_4NO_3$ and alanine as the nitrogen source for protein formation. The cell in such cases is confronted with the task of selection between the given alternatives or the use of all of the chemicals simultaneously, giving rise to sequential or concurrent utilization of nutrients. As might be suspected, the cell carries out one or the other course or both in the interest of maximum yield of protoplasm at the fastest rate.

Returning to the case of two sugar substrates for the purpose of illustration, there is of course a maximum rate at which some carbonaceous breakdown product of either sugar may be incorporated into protoplasm *via* a synthetic pathway. The prevalent concentrations of the sugars may be such that either sugar may provide the breakdown product (catabolite) in sufficient amounts as precursor for a synthetic pathway. The sugars "compete" so to speak, to provide the precursor. Due to kinetic limitations of the catabolic pathway, one of the sugars may not be able to supply the precursor as fast as the other sugar, and it is possible that the cell may choose to assimilate the "faster" sugar in preference to the "slower" one. With the aid of mechanisms developed through certain evolutionary processes to be discussed shortly, catabolism of the slower sugar is halted temporarily. Such a scheme of operation of selective operation of enzymatic pathways is economical since the energy required for transportation of the slow substrate across the cell boundary and its degradation as well as the energy of synthesis of the enzymes involved in transport and degradation processes are conserved. By the same line of reasoning, one may explain preferential assimilation of a nutrient which happens to be the catabolite of the less preferred nutrient.

In clarification of the concept of "competition" (or "interaction") between nutrients, it might be mentioned that there is conceivably no competition between nutrients having distinct roles in metabolism. For example, glucose should not be looked upon as a competitor for ammonium chloride as the former serves as a carbon or energy source whereas the latter serves to provide nitrogen for synthesis of nitrogenous organic matter of protoplasm.

The preferential use of the more economical substrate, which may be referred to as the "primary substrate," cannot conceivably continue indefinitely in the face of its continuing depletion from the medium as a result of growth. The survival characteristics of the microorganisms are such that rapid growth is to be continued at a maximum rate permissible within the constraints of the environment. This requires the cell to draw upon the "secondary substrate" for the supply of the growth factor as soon as it cannot be derived from degradation of the "primary substrate" at a rate commensurate with the rate of utilization of the growth factor for synthesis.

The metabolic pattern just discussed would obviously give rise to a growth curve of more than one cycle.[*] The first cycle is characterized by the preferential utilization of the primary substrate, exclusively at first, but concurrently with the secondary substrate after some point in the first growth cycle until complete exhaustion of the former substrate is realized. During the second cycle, growth is controlled solely by the secondary substrate. The resulting growth curve is complex with double cycle intervened by a transition phase when growth is slowed down due to physiological adjustments of the cellular machinery to cope with the secondary substrate instead of the primary.

A number of diphasic growth curves with the two sigmoid parts forming two steps were observed by Monod (7) during bacterial assimilation of interacting carbohydrates. The phenomenon was referred to as diauxie. The intervening transitional phase with an apparent growth lag should preferably be designated as the diauxic lag to differentiate this phase of complex growth cycle from the ordinary lag phase of a simple single cycle sigmoidal growth curve.

The above discussion might lead one to suspect that diauxic growth is inevitable if two or more growth controlling substrates are present. This of course would not happen if the concentrations of three competing nitrogen sources are so small that none of them can individually provide the nitrogen requirement for maximum growth rate. Also, substrates of similar chemical composition do not always compete. The investigations of Monod revealed that two carbohydrates, each of which may serve to be the source of carbon and/or energy, do not give rise to diauxie when present together in the growth medium.

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[*] Quantitative data on the mass of microbial protoplasm or numbers of organisms at different points in time of growth of a biological culture when plotted as a function of time give a sigmoid bacterial growth curve which may be looked upon as a cycle of different phases distinguishable from each other by differing growth rates.

A very pertinent question which arises is: how do microorganisms accomplish the selection of substrates? Microbes have developed for unnumbered generations in environments as competitive as those faced by other forms of life. While other species have not been able to survive when confronted with hostile environment, microbes have persistently proliferated by virtue of their capability to adapt to changing environments and reproduce rapidly. To compensate for unfavorable aspects of their environments, microbes have developed metabolic tricks. These are responses of enzyme synthesis and activity which tend to bring the intracellular environment into a condition suitable for rapid growth. This is a situation that exists as a result of evolution. Various chemical and physical forces in the environment determine the responses of enzyme synthesis and activity, and the enzymic make-up of the cell. The selection of substrate for metabolism only reflects the enzymic composition that has come into being for rapid growth and multiplication under the prevalent environmental constraints.

## Cellular Mechanisms for Regulated Uptake of Competing Substrates

In explanation of diauxic growth in the presence of glucose, it had been stated by Monod (7) that this substrate prevents the induction of the inducible enzymes of the secondary substrate as long as the former is present. Such a role of glucose was termed the glucose effect. Monod's hypothesis of enzyme induction does not explain how enzyme formation can be prevented in the presence of the secondary substrate which by itself or by one of its catabolites can induce the enzymes necessary for its degradation. There also remains a controversy regarding the inducibility and constitutivity of enzymes. Monod's hypothesis also does not directly explain the assimilation of an amino acid, such as arginine, in preference to an inorganic nitrogen source such as ammonium sulfate in a nitrogen controlling growth medium. It appears that prevention of formation of enzymes needed to synthesize the amino acid from ammonium sulfate is preferred in the interest of energy conservation, and it is plausible that this is accomplished by another mechanism which overrides the induction mechanism or deactivates it, thus nullifying the role of the inducer present. It has been shown that the catabolites and end products of enzymatic reactions do indeed control the synthesis and/ or activity of the enzymes responsible for formation of these compounds themselves. In the case of arginine containing medium, arginine, the end-product of the anabolic pathway for arginine synthesis, is said to inhibit the formation of ornithine transcarbamylase which catalyzes the formation of a precursor for arginine synthesis. This mechanism is referred to as end-product repression of enzyme formation. The mechanism is somewhat different in the case of a glucose-galactose substrate in that an intermediary catabolite in the glucose pathway, instead of an end product, may be responsible for controlling the formation of the inducible enzymes of the galactose pathway. The mechanism has been named catabolite repression. The mechanisms of end product repression or catabolite repression are put into action when the concentration of the end product or the catabolite provide the chemical potential adequate to stimulate the genes to modify the normal course of action (which is to synthesize enzymes in the presence of the inducer) and halt the enzyme making machinery.

While the repression mechanisms, which have received some experimental support, explain prevention of formation of enzymes, they do not explain how galactose enzymes present due to acclimitization of cells in galactose would be prevented from degrading this substrate notwithstanding the presence of glucose. It is suggested that the classical type of inhibition of the galactose enzymes by the glucose catabolites comes into effect to deactivate the enzymes while any de novo synthesis is prevented by catabolite repression. This mechanism of pure inhibition, commonly referred to as feedback inhibition or retro-inhibition, operates on existing enzymes to deactivate them and should not be confused with the repression mechanism which controls the formation or synthesis of enzymes. It has been indicated by molecular biologists that feedback inhibition is an emergency measure designed for swift control of enzymes present but not needed as contrasted with the sluggish gene-regulated repression mechanisms. All of the mechanisms discussed have received experimental support with regard to their roles in causing diauxic growth and sequential utilization of substrates either operating separately or jointly. The details of the mode of action of the catabolite in stimulating the genes into action and the mechanism of regulation of enzyme formation by the genes have not yet been revealed.

## Substrate Interactions in Sanitary Engineering Processes

### Status of Knowledge

The occurrence of sequential substrate utilization by heterogeneous cultures as encountered in aerobic biological processes in sanitary engineering has been demonstrated by several investigators. However, there have been conflicting reports of concurrent as well as sequential uptake of secondary substrates. The occurrences of sequential uptake have been variously attributed to "catabolite inhibition" -- meaning feedback inhibition -- or catabolite repression of the enzymes of the secondary substrate or both. Furthermore, glucose or its catabolites were not believed to affect the induction of enzymes of secondary substrates during the first phase of the diauxic growth. It has been implied that the glucose effect is peculiar to substrate systems comprised of glucose and other carbohydrates, and also that the glucose effect persists until this preferred substrate is completely exhausted from the growth media.

No hypothesis has been advanced to reconcile the conflicting reports of concurrent and phasic uptake of interacting substrates. As regards causative mechanisms, there remains no reported explanation as to when and why either feedback inhibition or catabolite repression or both would be operative. The contention that glucose does not affect induction of enzymes of the secondary substrates is untenable; it is hard to imagine why enzymes of the secondary substrate, which is not assimilated in the presence of the primary substrate, would be induced at all. The glucose effect or diauxic uptake is not always characterized by glucose being the antagonistic substrate, for there are reports of reversal of this role of glucose in dominant cultures where acetate and glucose served as carbon and energy sources. Finally, the theory of total rejection of the secondary substrate in the presence of the preferred one is difficult to comprehend. If this were to be true, then a fractional concentration of any preferred substrate would stop the metabolism of any secondary substrate, which of course cannot occur for it means starvation of organisms in the presence of substrates. In conclusion, it may be stated that little is known about the causative mechanisms underlying substrate interactions and the kinetics of assimilation of interacting substrates.

## Significance in Sanitary Engineering

The ramifications of sequential utilization of substrate components from heterogeneous wastes are of substantial significance in the management of the water quality of natural bodies of water, planning water pollution control programs, and the design of biological processes. Diphasic and triphasic oxygen utilization by biological sludges have been reported in multicomponent nutrient systems containing interacting substrates. The occurrence of double oxygen sags -- not predictable from the Streeter-Phelps concept of stream self-purification -- has been demonstrated in laboratory plug-flow models simulating stream flow and charged with two interacting substrates. Such studies point to the importance of consideration of sequential utilization in assessing and predicting the oxygen profiles in streams. Economic allocation of oxygen resources and usage of it is possible with a more accurate knowledge of the oxygen profile. This requires a knowledge of kinetics of sequential utilization. In biological processes, introduction of a waste stream with interacting substrate would result in complete or partial "leakage" of the secondary substrate resulting in drastic effects on the quality of the effluent from the process. Proper design and operation of biological processes would require diagnosis of potential sequential behavior and design of a treatment system from a knowledge of the kinetics of complex growth cycles.

## Scope of Further Studies

It appears that concurrent and sequential substrate utilization and growth are two important modes of action a microorganism is capable of carrying out as warranted by the environment and for the sake of most rapid growth and maximum yield of biomass. The mechanisms which become operative during diauxic phenomena are obviously not expressed in the absence of the interacting substrate. The genes play a significant role since the expression and suppression of physiological capabilities acquired through evolution and heredity are involved. Delineation of environmental and physiological factors which bring about the expression of the dormant capabilities of the cell to use substrates selectively are important in planning and predicting performances of pollution control systems. Use of a catalogue of interacting substrates to demonstrate the possibility of occurrence of the phenomenon in sanitary engineering processes is convenient for laboratory research, but such a procedure can hardly be of substantial aid to the practicing engineer who is encountering an enormously large number of chemicals in the waste. A rational approach would be to base predictions on analysis of biochemical pathways of the substrate system in terms of any possible interaction of pathways, energy requirements for different pathways, nature of enzymes involved, the characteristics of the rate controlling steps in alternative pathways, etc. To the author's knowledge, there is no report of such an approach which would lead to establishment of criteria for the occurrence of diauxie.

The kinetics of catabolite repression and feedback inhibition are obviously different, and the physiological and environmental factors which might result in either catabolite repression or feedback inhibition or both mechanisms taking effect need to be delineated.

Economical process design and stream water quality management demand a rational kinetic model for uptake of interacting substrates. Information on the effect of secondary substrate on the kinetics of utilization of the primary substrate and *vice versa* in comparison to the kinetics of utilization of each of the substrates as the sole carbon source would be helpful in achieving this objective. No effort has been directed in this direction. Information regarding the growth yield on individual substrates during the diauxic lag is of great importance in obtaining a process kinetic relationship. Such information could not be gathered by the investigators because of the batch reactors used in their experimentation in which the diauxic lag would last for only a limited period of time.

As mentioned earlier, the assumption that glucose does not inhibit induction of enzyme(s) of the secondary substrate is contrary to the hypotheses of the proponents of the theories of catabolite repression and needs to be examined.

It is known that the rate of cellular utilization of many substrates is controlled by the rate of enzyme mediated transport of the same through the cell wall, and there are also reports of repression and inhibition of enzymes taking part in the transport process. Although not indicated by any investigator, repression or inhibition of the cellular transport system may conceivably give rise to diauxic. Derivation of a kinetic expression may be greatly simplified if repression or inhibition is operative on the transport system which exhibits characteristics of a single enzymatic step.

Information on the topics enumerated above is prerequisite to the development of a kinetic model for biological processes designed to treat heterogeneous waste containing interacting substrates. This research was undertaken to provide some of this information.

# CHAPTER II

# REVIEW OF LITERATURE

## General

In order to establish the current status of knowledge pertaining to the causes, enzymatic mechanisms, and kinetics of diauxic metabolism and growth, a rather extensive search of the literature had to be undertaken covering areas of microbial metabolism and growth, environmental effects on microbial response, kinetics of simple and complex growth, and mechanisms of enzymatic and genetic control of cellular metabolism. The information compiled was expected to contribute towards a basic understanding of the sequential phenomena as well as to serve as an aid in formulation of the research methodology. The pertinent information has been disseminated in numerous symposia, lectures, conferences, and publications in diverse fields of science and engineering in all parts of the world. It was noted that very limited information on these topics of interest is available in the sanitary engineering literature.

## Biological Processes

Biological processes generally occur within limited environments where physical and chemical controls may be exercised and wherein chemical reactions catalyzed by enzymes secreted by bacteria, protozoa, algae, fungi, virus, and animals are in progress. The course and outcome of the process depend on the physiology, characteristic biochemical activities, and the proliferating abilities of the microbes and their genotypic and phenotypic capabilities to respond to environmental changes. For the purpose of analysis, the individual microorganisms may be considered as living micro-reactors in which raw materials (substrates and nutrients) are transported to be processed by degradative (catabolic) reactions into energy and degradation products. Some materials are used for the syntheses (anabolism) of polymeric substances like proteins, enzymes, nucleic acids, glycogens, lipids, etc. required for further growth and eventual fission (cell division) to yield two new and identical reactors. The end products are therefore energy, degradation products, and identical microorganisms.

Conceptually, the progress of biochemical conversion in the total process is a composite of the conversion rates in all individual organisms. However, due to the complexity of the reaction system -- which is to be discussed shortly -- there does not exist any comprehensive theoretical model[*] expressing the kinetics of conversion in an individual cell.

The classical approach towards derivation of a rate law for any biological process has been an empirical one, in keeping with engineering traditions. While the development of process kinetics models from the overall kinetics of the enzyme system has to await elaboration of complete enzyme sequences, evaluation of kinetic constants for enzyme reactions, delineation of interdependencies of different reactions and elucidation of other factors, rational models can be built to describe the macroeffects and behavior of the entire population of microbes in a process culture.

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[*] A model is simply a symbolic form in which physical and chemical principles are expressed. It may be an equation that is derived by consideration of the pertinent scientific principles, operated on by logic, and modified by experimental judgment (8).

One such approach is to relate fermentation kinetics to kinetics of growth of the entire culture. Gaden (9) has discussed this approach where kinetics of growth and product formation (or substrate utilization) are closely related. Another approach involving the application of the absolute reaction rate theory to the total biological system has been discussed by Aiba, *et al*. (10). Both approaches are similar since the derivations of the rate laws are based on consideration of an overall reaction scheme in which process reactants are converted to final products after passing through a transient state of high potential energy.

Because the performance and control of any biological process are dependent on the driving forces for transport through the cell wall, the permeability of cell membranes to penetration, cellular metabolism, growth, and modification of these various cellular processes with environmental change, some discussions of these topics were considered warranted.

## Cellular Transport of Nutrients

The rate of transport of nutrients from the medium to the cell boundary is controlled by the physical forces of diffusion, convection, and mixing. However, the transportation of nutrients from the cell boundary to the cytoplasm is frequently accomplished with the help of enzymes.

Penetration by nutrients through a cell wall often requires degradation of macromolecules by *extracellular enzymes* liberated from within the cell by a mechanism not completely understood at the present time. Lampen (11) has postulated that exoenzymes are formed on the cytoplasmic membrane and are excreted through the cell wall during growth and elongation of the membrane, aided by simple diffusion or mediated transport in the presence of an autolytic enzyme called autolysin.

Following this action which is frequently hydrolytic in nature, absorption and transport across the cell wall may be accomplished either by active transport characterized by concentration within the cell against a gradient and coupled with the expenditure of metabolic energy, or by passive transport by simple diffusion. Passive transport, however, plays a minor role since the cytoplasmic membrane is very poorly permeable even to hydrophilic compounds and most metabolic intermediates generated in the cytoplasm stay within the cell and hardly diffuse out through the membrane serving as an osmotic barrier (12).

Active transport is brought about by ectocellular enzymes seated on the cytoplasmic membrane (13). These enzymes, called permeases by Rickenberg, et al. (14) and Monod (15), regulate the interactions between the internal and external environments by controlling the entry of the nutrilites into the organism. The permease system is comprised of functionally specialized proteins which are stereospecific toward substrates and distinct from metabolic enzymes (12). Therefore, according to the permease model (16-24), nutrients are selectively transported through the mediation of stereospecific enzyme-like proteins involving the expenditure of energy.

Active transport is also characterized by an entrance reaction mediated by specific enzyme-like substances also called permease, and an exit reaction for the simultaneous emanation of nutrients catalyzed by exitase (19-23). While the permeases obey classical laws of enzyme kinetics (25) and require energy, opinions differ regarding similar properties of the exitase system (19-23). The important function of the permease system is its great ability to accumulate substrates within the cell in concentrations far exceeding those found in the external medium (20-23). The steady state concentration of a substrate within a cell can be considered to represent a balance between the rate of uptake of the same and the rate of its loss to the medium (20).

In light of the above discussion, it is apparent that the course and kinetics of a biological process may be dictated by the permeases since many of the growth-promoting and growth-inhibiting effects of components of complex media may be due to specific interactions at the cell membrane. A review of the subject (26) points to an important aspect; i.e., the constitution of the permease system is dependent on the substrate to be transported rather than on the microbial species.

## Enzymes and Metabolism

It has been pointed out previously that nutrition of bacteria by nutrients and substrates fills two needs: materials for construction of protoplasm and energy stored in energy-rich compounds for the endergonic physical and chemical processes of the organism. The process of production of protoplasmic building blocks and energy by catabolism, as well as the process of assembling the building blocks into protoplasm by anabolism are so intertwined that this total process of biochemical transformation of nutrient material within a cell is frequently referred to as *metabolism*. Each line of metabolism requires a coordinated system of several enzymes to bring it about. The number of enzymes, organized in straight or cyclic chain systems, is determined by the number of successive steps involved in the whole process. Thermodynamically, each line of metabolism represents a multibarrier free-energy profile punctuated by energy peaks and valleys. The energy difference between the initial reactant and the final product is released or supplied (depending on the direction of reaction in the reversible chain) in graduated quantities after each energy peak is crossed. An extended discussion of profiles of free-energy, enthalpy, and entropy may be found in a treatise by Lumry (27).

In the metabolic process, the substrate molecule is acted on successively by all the enzymes in tandem and arranged in a definite sequence forming a *pathway* so that a final product of the sequence is formed to be passed on to another metabolic line. Thus the pathway contains a number of enzymes whose specificities form an unbroken chain such that the product formed by each enzyme falls within the range of specificity of the following enzyme. Naturally, if any one of the enzymes is missing or inhibited as might happen in an environment, the process is blocked at that point and the whole system becomes inoperative. Finally, it should be emphasized that the cellular metabolism as a whole is brought about by one great network-like multi-enzyme system. The different pathways are interlinked, and breakdown products of one catabolic pathway may serve as the substrate of an anabolic pathway. Depending on the environment, especially with regard to the extracellular substrate concentration, the chain reaction may move in either the forward or the reverse direction. The whole pathway reaches a steady state or a dynamic equilibrium when each stage in the pathway is near but not quite at its own equilibrium and the intermediate metabolites remain at low concentration with constant formation and breakdown (28-30).

Classification of the enzymes or enzymatic pathways as degradative (catabolic) and biosynthetic (anabolic), according to the degradative or synthetic function of the enzyme or pathway, is not adequate because some enzymes at the junctions of catabolic and biosynthetic pathways might serve as both. Also, due to the reversible nature of the enzymatic reactions, some pathways may be used for catabolism or anabolism. Davis (31) has defined amphibolic enzymes to be those which fulfill both anabolic and catabolic functions. Accordingly, an amphibolic pathway is a pathway which may be used for catabolism or anabolism. This review of the regulation of enzymes and enzyme formation indicates that, due to their strategic importance, the amphibolic enzymes and pathways frequently become the subjects of cellular regulation.

Operation along a particular metabolic line and its associated kinetics is very much dependent on environmental factors. Depending on these environmental conditions, one of several possible products may be formed via one of several routes. In some cases the substrate has been shown to be degraded by the simultaneous use of several alternate routes (32). The chemical and physico-chemical constitution of the environment may affect the affinity of the enzyme system for the substrate and accelerate or decelerate the velocity of transformation. The formation of enzymes and the amounts of each of these catalysts are regulated by the nucleic acids, the very synthesis of which is dependent on enzyme catalyzed reaction.

The picture which emerges from the foregoing is that of a vast network of interdependent reaction systems which may remain entirely or partly operative at maximum or reduced velocity at any time depending on environmental factors. This complexity, coupled with the dearth of information regarding enzyme sequences and their thermodynamic properties, makes it extremely difficult to formulate rate laws of substrate conversion or product formation from analysis of the reaction systems.

## Growth

### Growth of Individual Cells

Growth of a cell reflects the harmonious functioning of the geared enzyme system leading to the addition of macromolecular products to the protoplasm. However, addition of protoplasmic mass and growth of an organism do not go on indefinitely and, after a characteristic size is reached, the cell divides due to hereditary and internal limitations. It has been hypothesized that the decrease in surface area to volume ratio of the cell with increase in size leads to the necessity of segmentation of the cell, although evidence also exists in opposition to this hypothesis (13). With many species, the rate of growth of an individual cell may follow a sigmoid growth curve, the growth being least just prior to and after cell division (13, 33-34). The generation time of organisms has been variously defined as the time interval between the fission of the nucleus of the mother and the daughter cell or the time period between the appearance of the daughter and granddaughter cells (35). Generation time, however, is not constant even for the organisms within a clone since the time of duplication of the genes within an organism varies as a chance event (13). The distribution of the generation times has been variously claimed to fit a Yule's distribution (13), a normal or the Gaussian distribution (36,37), and skewed Pearson Type III or Type V distribution (35). Further, the variability of the cell division rate (number of cell division/unit time) can be modified by environmental conditions (36). Therefore, organisms of identical hereditary characteristics are capable of exhibiting generation times varying within a range even when subjected to identical environments.

## Development and Growth of Microbial Culture

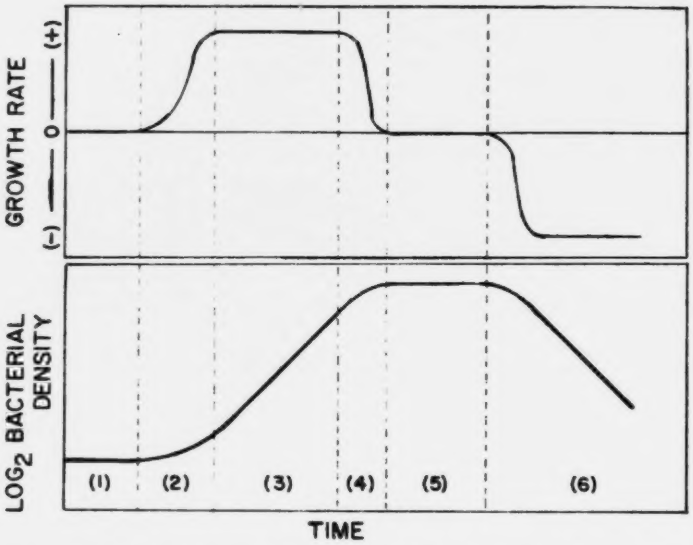
The process of fission occurring in the mother cell would be repeated in the daughter cells, the granddaughter cells, and so on, resulting in an increase in both the mass of protoplasm and the numbers of organisms in the culture. Such is the process of development of microbial culture. It may be imagined that the daughter cells and the cells of the subsequent generations should divide in unison at the end of each generation time since all cells share the same heredity. The increase in protoplasmic mass (biomass) and numbers of organisms would then be characterized by discrete positive shifts following and preceding a constant level. However, due to variability of generation time, all of the individuals in the population rarely go through their fission cycles in concert to exhibit this type of synchronous growth as envisioned above. In heterogeneous culture, of course, the possibility of occurrence of synchronous growth cannot be perceived.

The growth of the population, i.e., the growth of the microbial culture, continues in smooth fashion until a characteristic maximum is reached when further growth is not possible due to limitations of food and other factors. Although exceptions have been noted, the time-course of increase of numbers of cells or biomass frequently follows a sigmoid or S-shaped function which would be repeated if a few microbes are transferred (subcultured) in a fresh medium. Analysis of the sigmoid curve or the so called microbial growth curve reveals a complex and characteristic cycle of physiologic and metabolic phenomena. The phases of the cycle (which have been named somewhat differently by different authors) are conveniently separated at points of the growth curve denoting changes of slope (7, 13). The characteristics of the various phases of growth (see Figure 1) as defined by Monod (7, 38) are summarized (13) below.

1. Lag phase: Adaptation to a new environment; very long generation time; growth rate null; duration longer for species with longer generation time in exponential phase; length of lag varies according to the phase from which inoculum is derived and decreases with increase in inoculum size; lag period does not affect course of subsequent phases; cell size and rate of metabolic activity maximum at lag phase; rate of multiplication lags much behind the rate of production of protoplasm; cell size is maximum.

2. Acceleration phase: Decreasing generation time and increasing growth rate.

3. Exponential phase: Minimal and constant generation time; maximal and constant growth rate; maximum rate of substrate conversion and product formation; achievement of steady state as indicated by nearly constant ratio of DNA/cell, RNA/cell, protein/cell, enzymes/cell, constant cell density and constant and minimum cell size; growth rate in this phase is a property of the particular species.



(NUMBERS ON THE FIGURE BETWEEN VERTICAL DOTTED LINES REFER TO PHASES AS DEFINED IN TEXT)

FIGURE I. SCHEMATIC DIAGRAM SHOWING THE GROWTH PHASES AND VARIATION OF GROWTH RATES BETWEEN PHASES (AFTER MONOD (7))

4. Retardation phase: Increasing generation time and decreasing growth rate due to gradual decrease in food concentration and increased accumulation of toxic metabolites; increasing death rate.

5. Stationary phase: Exhaustion of nutrients, high concentration of toxic metabolites, maximum physical crowding or M-concentration effect (39); multiplication rate balanced by death rate; growth rate null.

6. Phase of decline: Endogenous metabolism (40), high death rate, lysis, regrowth or cryptic growth (41,42); multiplication overbalanced by death rate resulting in negative growth.

With reference to Figure 1, it should be mentioned that increases in biomass concentration do not necessarily coincide with the rate of increase of cell concentration (numbers/unit volume) at all phases of growth, although such coincidence is the characteristic of the exponential phase where cell size remains nearly constant.

## Mathematics of the Simple Growth Curve

Biologists have been inspired to develop mathematical expressions for growth ever since the presentation of the doctrine of human population growth by Malthus (43,44). The sigmoidal curve, proposed empirically in the Malthusian doctrine, is described by a mathematical ("logistic") function proposed by Verhulst (45) and Pearl and Reed (46). The "logistic" function has been adopted by some (47,48) as the equation for microbial growth, the logic for acceptance being that the function represents all phenomena underlying population growth. It is known that the growth curve of a bacterium or of a bacterial population, of human or animal limbs, of population growth of rats, rabbits, swine, pigeons, horses, doves, flies, chicken embryos, pre-pubertal or post-pubertal growth in humans and rats (49), as well as the rate curves of numerous phenomena of physics, chemistry, biology, and human society are sigmoid in nature. Thus the mere fact that a microbial growth curve is of sigmoid shape does not prove that growth is analogous to another phenomenon with the same type of development, although this assumption has sometimes been made. It has been aptly pointed out by some biologists (31,50) that the analogy is mathematical and not a physico-chemical one. Since this equation does not express the biochemical phenomenon of growth, and since the constants involved have no physiologic significance, the equation is not a mechanistic model of microbial growth. An equation which gives mere coincidence of numbers is of no theoretical use to the microbiologists or the process engineer unless it depicts or suggests the modus operandi of the underlying metabolic phenomenon.

As it can be imagined, construction of a theoretical model of microbial growth from consideration of all of the metabolic peculiarities of all phases of growth is a formidable task. Beran (51) stated that it is difficult to express variation of growth rate by a simple equation since each of the metabolic processes in the cell has a different kinetic course and affects the overall growth rate. While limited theoretical analyses have been performed for the lag phase (38,52), stationary phase and phase of decline (42,53,54), much more effort has been expended for the derivation of a theoretical expression for growth during the exponential phase. Since the exponential phase is of special importance to the process engineer by virtue of the associated maximal metabolic rate, it is appropriate to discuss the mathematics of this phase in detail.

## Mathematics of Exponential Growth

The growth of a culture during the exponential phase is said to be *balanced* (55) to signify growth over a period of time during which all constituents of the organisms are, mathematically speaking, increased by the same factor. Moreover, balanced growth is said to be *unrestricted balanced growth* when medium constituents are not limiting for the rate of growth attained. Conversely, *restricted balanced growth* exists when the growth rate is limited by a constantly maintained limiting concentration of a single constituent of the medium (13). For balanced growth under restriction of a single *limiting*[*] substrate or nutrient, the *exponential growth rate*[**] tends to depend on variations in the concentrations of this limiting factor only in dilute media. A mathematical model describing the effect of the concentration of the limiting nutrient on the *specific growth rate*[***] of microorganisms has been proposed by Monod (7, 38). As stated by Monod (38), this simple law expresses the growth rate

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[*] Any one of the essential nutrients in the growth medium is a potential limiting factor. Composition of the medium may be such that concentrations of all essential nutrients are in large excess compared to that of one of them which then becomes the limiting nutrient and remains so as long as its concentration is low enough to eliminate interference from other potential limiting factors (pH, oxygen concentration, etc.) or nutrients (38). Change of concentration of the limiting nutrient, by definition, should affect growth, substrate consumption, or product formation.

[**] Growth rate during the exponential phase is the slope of the straight line in a plot of a logarithmic growth curve (13).

[***] Slator (56) first proposed the following mathematical formula for the determination of the *specific growth rate constant* or the *velocity coefficient*:

$$k = \frac{2.303}{\theta_2 - \theta_1} \log \frac{X^{o}_{\theta_2}}{X^{o}_{\theta_1}} \qquad (1)$$

where $k$ = specific growth rate ($\text{time}^{-1}$); $X^{o}_{\theta_1}$ = organism concentration at time $\theta_1$; and $X^{o}_{\theta_2}$ = organism concentration at time $\theta_2$. (Continued) in the exponential phase as a function of the concentration of the limiting nutrient.

$$k = \frac{k^m X_\theta^n}{K + X_\theta^n} \qquad (3)$$

where $k^m$ = maximum limit of $\underline{k}$ for increasing concentrations of the growth limiting nutrient, and commonly called the maximum specific growth rate

$X_\theta^n$ = concentration of limiting nutrient at time $\theta$

$K$ = concentration of nutrient when $\underline{k} = k^m/2$, and commonly referred to as the *saturation constant*.

It is important to note that $k^m$ and $\underline{K}$ are influenced by the degree of complexity of the medium and the physico-chemical nature of the environment. Monod (7,38) also proposed that *total growth*, $X_t^o$, bears a linear relationship with the initial concentration, $X_o^n$, of the limiting nutrient. Thus

$$X_t^o = X_{max}^o - X_o^o = Y^o X_o^n \qquad (4)$$

where $Y^o$ = *observed* or *apparent yield coefficient* assumed to be constant.

Due to the empirical adoption of Equation 3, often referred to as

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[***] (Concluded) It is evident that $\underline{k}$ is the slope of the straight line plot of the logarithmic growth curve, and when computed from equation (1), it represents the average value of growth rates during the interval of time $(\theta_2 - \theta_1)$. By proposing Equation 1, Slator in effect proposed the *primitive* of the following differential equation expressing the basic law of microbial growth:

$$\frac{dX_\theta^o}{d\theta} = kX_\theta^o \qquad (2)$$

the Monod Growth Kinetic Model, numerous criticisms have appeared in the literature regarding its adequacy to describe microbial growth. The objections are briefly enumerated as follows:

1. The model is not expected to adequately describe growth in phases other than the exponential phase (57).

2. The model is too simple to describe complex metabolic processes giving rise to growth (58,59).

3. Death rate during growth phase is neglected (38).

4. Implies constant generation time.

5. Interference of toxic metabolic products and modified environment towards the latter part of the exponential phase may become growth controlling instead of the limiting nutrient (58). Reduction of growth rate may be brought about by a variety of physico-chemical effects other than the concentration of limiting nutrient.

6. The model is not expected to describe growth when controlled by the rate of transport across the cell wall (60).

7. The model is inadequate to describe batch growth, since it does not correct for the effect of toxic metabolites, death, etc.

8. Equations for batch growth curves based on the Monod equation are not amenable to easy mathematical solution (46,61,7).

9. Assumption of constant growth yield with respect to limiting nutrients is not valid (57-59, 62-68).

10. At large concentrations of limiting nutrients, *k* may decrease instead of approaching $k^m$ due to inhibition by excess concentration or osmotic effects (58).

11. The model is incapable of describing growth and metabolism in completely mixed, continuous cultures (57,59,62-65,67).

Figure 2 shows a plot of specific growth rate, k, as a function of concentration of the limiting nutrient (glucose). The curve closely approximates a rectangular hyperbola described by Equation 3. Monod idealized the curve by drawing it through the origin, although the data would permit the curve to originate at some positive value of the abscissa, $X_m^s$, thereby implying that there is no small concentration of glucose below which growth is impossible. As pointed out by a number of authors (13,40,59,62-71), this assumption is invalid in many cases and modification of Equation 3 would be required. This aspect of the Monod growth equation is discussed in a later section.

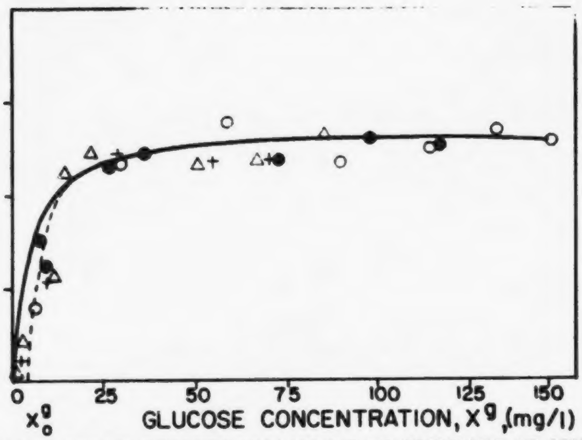
Owing to the aforementioned limitations of the Monod equation, various other growth kinetic hypotheses by Teissier (72), Novick and Szilard (73,74), Spicer (75), Moser (76,77), Ierusalimsky (78), Contois (79), Takehiko (56), Powell (60), van Uden (80), Saidel (81), and Leudeking and Piret (82) have been used in preference to Equation 3. The Teissier equation,

$$\frac{dk}{dX^s} = c(k^m - k) \quad . \; . \; . \; . \qquad (5)$$

whence

$$k = k^m(1 - e^{-cX^s}) \quad . \; . \; . \; . \qquad (6)$$

empirically proposed before Monod to describe a first order increase in k, was preferred by Lipe (62) and Schulze (63-65), although the latter author had earlier concluded that variation of k follows a "saturation



(NOTE THAT THE DATA PERMITS THE CURVE TO ORIGINATE AT SOME POSITIVE VALUE $X^g_o$ ON THE ABSCISSA. $X^g_o$ REPRESENTS THE REQUIREMENT FOR ENERGY OF MAINTENANCE OF THE CELLS AND NO GROWTH IS POSSIBLE AT GLUCOSE CONCENTRATIONS BELOW $X^g_o$)

FIGURE 2. GROWTH RATE OF *E. coli* AS A FUNCTION OF GLUCOSE CONCENTRATION (AFTER MONOD (7))

or absorbtion" curve (83) similar to the Monod equation. Novick and Szilard (73,74) used a zero order increase of k with respect to the concentration of the limiting substrate. The equations of Spicer, Takehiko, Leudeking and Piret, and Saidel take into account the effect of secreted toxic metabolites and death on growth rates. Takehiko's equation is claimed to describe all of the phases of the growth curve if the "critical cell concentration" and the "coefficient of consumptive activity" can be estimated. Powell, van Uden, and Saidel considered mass transfer rate through the cell wall to be an important factor and suggested the incorporation of factors representing diffusivity, permeability, and surface structure of the cell. Ierusalimsky paid special attention to the effect of cell concentration in causing a decrease in specific growth rate and his equation involves philosophic and unmeasurable constants, e.g., "metabolic coefficient," "trophic coefficient," etc. which are of nebulous significance. Contois introduced a parameter, p, (see Equation 7) in the Monod equation to account for the decrease in k at high population density, or

$$k = \frac{k^m X_\theta^n}{pK + X_\theta^n} \quad . \; . \; . \; . \qquad (7)$$

where p = population density.

The equation of Moser (see Equation 8) can be reduced to the form of Monod's equation if the constant, λ, is unity.

$$k = \frac{k^m}{1 + K(X_\theta^n)^{-\lambda_1}} \quad . \; . \; . \; . \qquad (8)$$

## Kinetics of Growth in Heterogeneous Culture

Since the classical works of Butterfield and co-workers (84,85), the data of many researchers (86-88) have conclusively shown that the growth curve in heterogeneous culture follows the same sigmoid function as observed with pure cultures. This is not very surprising since the dominant species surviving under the given environmental constraints express common physiological capabilities under the dictates of the physico-chemical factors of the growth medium; in this respect a heterogeneous culture is akin to a pure culture. Since the growth curves of heterogeneous (or dominant) and pure cultures are similar, it might be anticipated that the kinetics of growth in both cases could be described by a similar mathematical formulation. In fact, Garrett and Sawyer (87) have reported that the kinetics of substrate utilization by mixed cultures were the same as had been observed for pure cultures of bacteria.

Since the presentation of the mathematical formulation of the sigmoid growth curve by Fair and Moore (86), considerable effort has been expended by Sanitary Engineers to arrive at simplified versions of these equations. Following the suggestion of Fair and Moore regarding approximation of their continuous function by "two-phase" discontinuous functions, Garrett (89), Garrett and Sawyer (87), and later McCabe (90) have elaborated on the mathematical forms and methods of determination of the two rate constants which are said to approximate the growth curve. However, the pseudo-transition point or the point of discontinuity between the two phases represents values of substrate concentrations which are much higher than the observed substrate concentrations; better agreement between observed and predicted substrate concentrations can be obtained if the predictions are based on the Monod equation (91). The other equations which have found application in heterogeneous cultures of high microbial density have been recommended by Gram (92), McKinney, *et al.* (93), and McKinney (94).

While these equations may be suited for engineering design of biological processes to be operated at a particular growth phase, the fact remains that these equations describe specific growth rate as a discontinuous and unrealistic function of the food concentration and are merely approximations of the Monod equation which has been shown to satisfactorily fit data on growth and metabolism by heterogeneous cultures (61,91,95-99).

## Evaluation of the Monod Growth Kinetic Model

While objections to Monod's assumptions of constant growth yield, of disregarding the effect of the altered environment, and of toxic metabolites on growth have some merit, the other adverse criticisms do not appear to have sufficient basis. For example, transport limited growth is expected to be described by Equation 3 since permease controlled transport and growth obey the same kinetic law, namely the enzyme kinetic model of Brown (101), Henri (102), Michaelis and Menten (103), and Briggs and Haldane (104), which serves as the basis of the Monod model. Monod justifiably neglected the death rate since death rate in the exponential phase of dilute cultures is negligible (13,28), informations regarding death rates are inconclusive, and accurate techniques of measurements are not available. Thus Monod's specific growth rate can be viewed as the net growth rate which is of most practical importance. The equations for batch growth and nutrient assimilation derived by Monod (7) are admittedly cumbersome and are not without tedium for mathematical manipulation. However, computer methods of solution by nonlinear least squares analysis (100,105) or solution by graphical techniques (106) are now available.

From the foregoing it may be concluded that the equations proposed to replace the Monod growth kinetic equation have no more theoretical basis than the latter. Many of the observed disagreements are due to attempts to fit data on growth during the acceleration and retardation phase or the phase of decline in disregard of the fact that the model was proposed for the exponential growth phase. While empirical equations have limited applicability due to the lack of firmer scientific basis, rigorous theoretical analysis in line of the approaches of Deans (107) and the concept of "total integration" and "network theorem" of Dean and Hinshelwood (52) remain incomplete by the authors' own admission. On the other hand, the Monod growth kinetic equation is acceptable because it is rational and firmly based on the law of growth by binary fission and the universally accepted Michaelis-Menten law of enzyme kinetics. Furthermore, the equation is simple relative to the complex biochemical phenomena it describes and the constants of the equation have interpretable physiological significance. Finally, the model is of proven value in analyses and prediction of the kinetics of fermentation (10,28) and sanitary engineering processes (71,91,95-98,100).

## Physiological Significance of Constants of the Growth Equation of Monod

As shown later in Chapter IV, the saturation constant, K, is related to the Michaelis constant, $K_m$. The value of K indicates the affinity of the bacterial enzyme system for the substrate and provides a guideline for comparison of the growth supporting capacities of substrates (108). Jacob and Monod (25) have indicated that the reciprocal of the saturation constant is a measure of the apparent affinity of the organisms for the substrate. It has been further suggested by Monod (38) that K bears a

> ...distant relation to the apparent dissociation constant of the enzyme involved in the first step of the breakdown of a given compound [and that] ...one might expect the K values to be lower than the corresponding values of the Michaelis constant of the enzyme catalyzing the reaction.

The significance of $k^m$ lies in the fact that it is a very useful parameter for comparing the efficiency of a series of related compounds as the source of an essential nutrient (38). From the viewpoint of interpretation of diauxic phenomena, a more important significance of $k^m$ is best expressed in the following statement of Monod (38,34):

> There is no doubt that it ($k^m$) is related to the activity of the specific enzyme systems involved in the breakdown of the different compounds, and it can be used with advantage for the detection of specific changes, e.g., hereditary variation affecting one or another of these systems.

The yield coefficient, $Y^o$, which may be defined as growth yield per unit quantity of substrate utilized, or the product yield per unit quantity of substrate assimilated or biomass synthesized is a function of the chemical nature of the substrate, the type of microbial species (i.e., the enzyme system) involved and environmental factors. Moreover, growth yield, which gives rise to biological solids production, is not dependent on the Gibbs' free energy of oxidation of the substrate (13, 109,110,111) as contended by some (112,113). The implication by McKinney (114,115) that growth is related to the enthalpy change of substrate oxidation has been shown to be questionable by Schroeder and Busch (111).

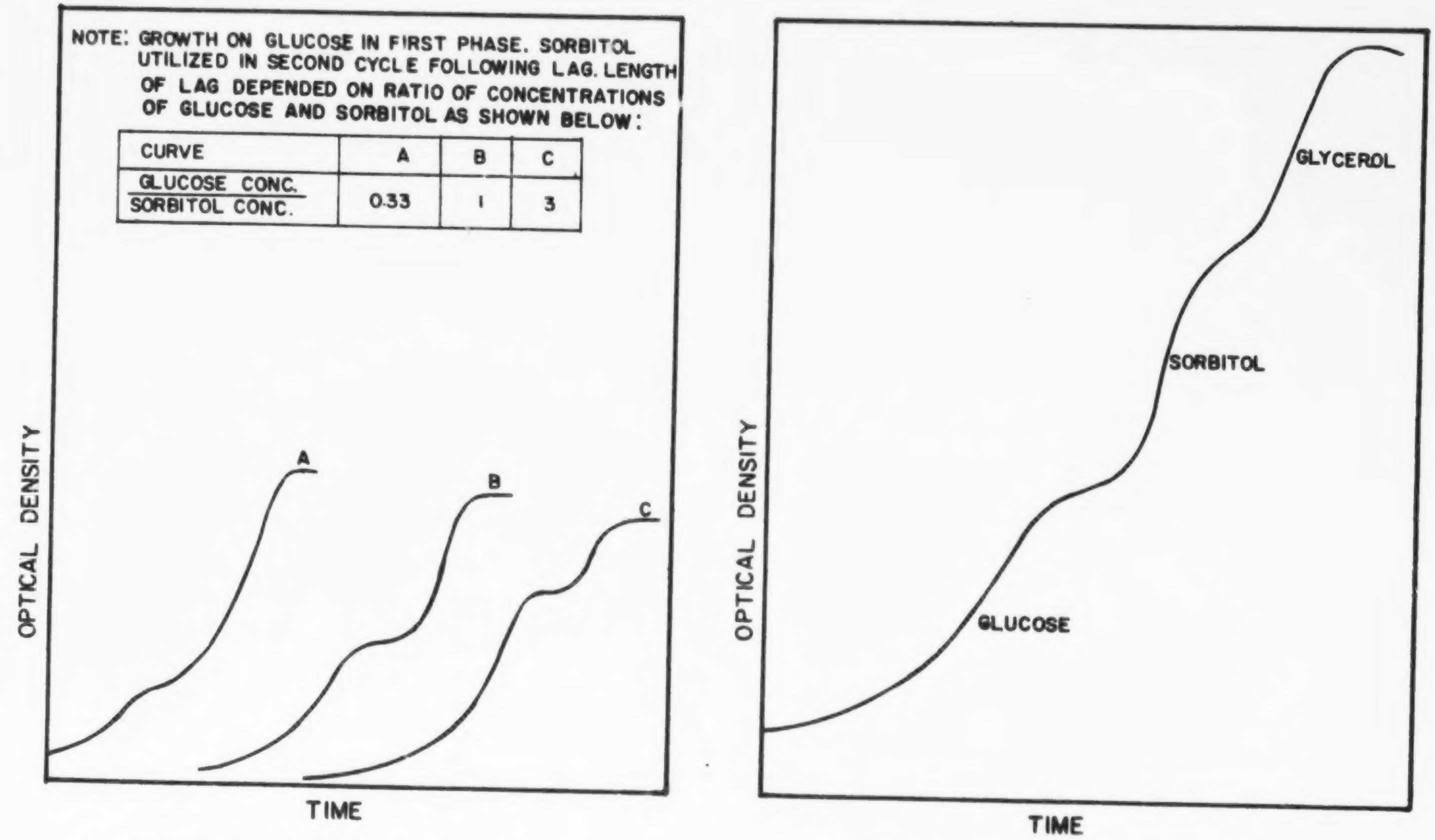
Had this hypothesis been valid, the same growth yield would be expected from all of the ketohexoses which is not true. Bauchop and Elsden (109) and Hetling and Washington (110) have shown that growth yield is dependent on the yield of ATP per mole of substrate assimilated, a quantity not necessarily related to theoretical COD or free energy or enthalpy change of substrate during metabolism. Cell yields may be different even for the same organism utilizing the same substrate depending on the environment (aerobic, anaerobic, etc.) and the metabolic pathway used. Furthermore, the growth yield is higher in complex media because some of the monomers and building blocks for protoplasm are readily available and more ATP can be diverted for polymer synthesis. Hetling and Washington (110) have concluded that mixed culture of organisms gives a higher yield on a substrate than a single species.

In light of the above exposition, it may be concluded that the concepts of dependence of growth yield on the various factors envisioned by Placak and Ruchhoft (116), Sawyer (117), McCabe and Eckenfelder (118), McKinney (94), Helmers, _et_ _al_. (119), Huekelekian, _et_ _al_. (120), and Hoover, _et_ _al_. (121) are of questionable validity. The yield coefficient is indicative of the fraction of net ATP production (which is dependent on environmental factors and the nature of microbial species) available for synthesis of polymers of biomass as well as on that fraction diverted for other energy requiring functions.

## Complex Microbial Growth Cycle

The discussions on microbial growth and metabolism put forth up to this point have been in reference to biological cultures exhibiting simple growth cycles as depicted by a sigmoid curve. However, there have appeared numerous reports showing stepwise fermentation of substrates and yielding complex growth curves which appear to be a composite of two or more sigmoid curves in series and connected by step-like phases or pauses (see Figures 3A and 3B) during which the growth rate passes through a minimum and even becomes negative (38).

The phenomenon was first reported in 1901 by Dienert (122) who observed that glucose had an inhibitory effect on the uptake of galactose and the formation of "galactozymase" by yeast. Stephenson and Yudkin (123), in 1936, confirmed the observation of Dienert (122). Following these investigations, Monod (124), in 1941, discovered this phenomenon with bacteria growing in medium containing certain mixtures of two carbohydrates and where the organic source was growth limiting. Growth was characterized by a double growth cycle (*croissance double*) or a *diauxic* and was suggestive of *attaque differentielle* of one carbohydrate initially from the mixture. Recognizing the distant similarity of the phenomenon of diauxic with the *Pasteur effect*, according to which pyruvate and lactate production by glycolysis is forsaken by facultative organisms in preference to a more rapid growth in an aerobic environment due to inhibition by oxygen of some key enzymes for pyruvate formation, Monod (38) suggested that preferential utilization is attributable to an inhibitory effect of one of the carbohydrates of the mixture on the formation of the enzymes necessary for attacking the other. From an analysis of the results of Karström (125), and following his hypothesis (125,126) that microbial assimilation of certain compounds requires the formation of some *adaptive* enzymes, Monod (7) classified his test carbohydrates into two classes as follows:



NOTE: GROWTH ON GLUCOSE IN FIRST PHASE. SORBITOL UTILIZED IN SECOND CYCLE FOLLOWING LAG. LENGTH OF LAG DEPENDED ON RATIO OF CONCENTRATIONS OF GLUCOSE AND SORBITOL AS SHOWN BELOW:

| CURVE | A | B | C |
|---|---|---|---|
| GLUCOSE CONC. / SORBITOL CONC. | 0.33 | 1 | 3 |

A. DIAUXIE ON GLUCOSE & SORBITOL
(AFTER MONOD (38))

B. TRIAUXIE ON EQUIMOLAR CONCENTRATION OF GLUCOSE, SORBITOL AND GLYCEROL
(AFTER MONOD (34))

FIGURE 3. SCHEMATIC REPRESENTATION OF MULTICYCLE GROWTH CURVES OF *E. coli*

Class A - glucose, fructose, mannose, mannitol,
(preferred substrates) and saccharose
Class B - galactose, arabinose, xylose, rhamnose,
(spared substrates) sorbitol, dulcitol, inositol, maltose,
and lactose

Karström's data had indicated that, in contrast to Class A carbohydrates, degradation of Class B carbohydrates proceded only when the cells were previously "trained" in the carbohydrate which was to be utilized later. Accordingly, it was concluded that Class B carbohydrates require the formation of *inducible* or *adaptive* enzymes in the presence of the particular carbohydrate in question before degradation can occur. On the other hand, no induction was necessary for breakdown of Class A carbohydrates because the enzymes involved were *constitutive* as opposed to being *adaptive*, and as such these enzymes are always present. Monod's data (7) further revealed that

a) any of Class A carbohydrates + glucose + *B. subtilis* → no *diauxie*;
b) any of Class B sugar + glucose + *B. subtilis* → *diauxie*; and
c) Class B sugar + Class B sugar + *B. subtilis* → no *diauxie*.

From the above type of analysis, Monod (7,34,38) concluded that diauxic or diphasic growth and substrate utilization are caused by inhibition of induction of the adaptive enzymes or suppression of the adaptive enzymes necessary for breakdown of Class B carbohydrates in the presence of glucose or any other Class A carbohydrate. It is to be noted, however, that the above classification of carbohydrates applies only to the species *B. subtilis* and *E. coli*. Some compounds may have to be classified as Class A or Class B according to the microbial strain under test. Whereas prior training in Class B does not suppress diauxie when exposed to both A and B, absence of training accelerated the diauxie (7,34). The length of the diauxic lag phase intervening between the two consecutive exponential phases is a function of the ratio of Compound A and Compound B; the maximum lag period occurring when the ratio of concentration of A to concentration of B equals unity (34). The length of the pause is dependent on the nature of the Carbohydrate B. Thus Monod (7) observed a long pause for the glucose-dulcitol system and a short pause for the glucose-galactose system. The length of the pause gradually decreased with the increase in concentration of the Class B compound.

The above discussion should not be construed to mean that the phenomenon of phasic growth is restricted only to double growth cycles, for triauxic growth or triauxie has also been observed by Monod (34) on a medium with glucose, sorbitol, and glycerol (see Figure 3B).

Glucose Effect. Observations of the role of glucose in suppressing the microbial assimilation of carbohydrates and other organic compounds have been so numerous (26,88,127-138) that diauxic growth and metabolism have often been explained away as the "glucose effect" which is actually a symptom rather than the cause of diauxie. This type of crude explanation is untenable since reports exist where glucose yields its familiar antagonistic role to organic acids of the tricarbxylic cycle (139,140). The glucose-effect is not evidenced in organisms which have lost the capacity to synthesize the inducible enzymes of the Pentose-Phosphate Cycle (or the Warburg-Dickens-Horecker pathway)(see Figure 4) (140-142).

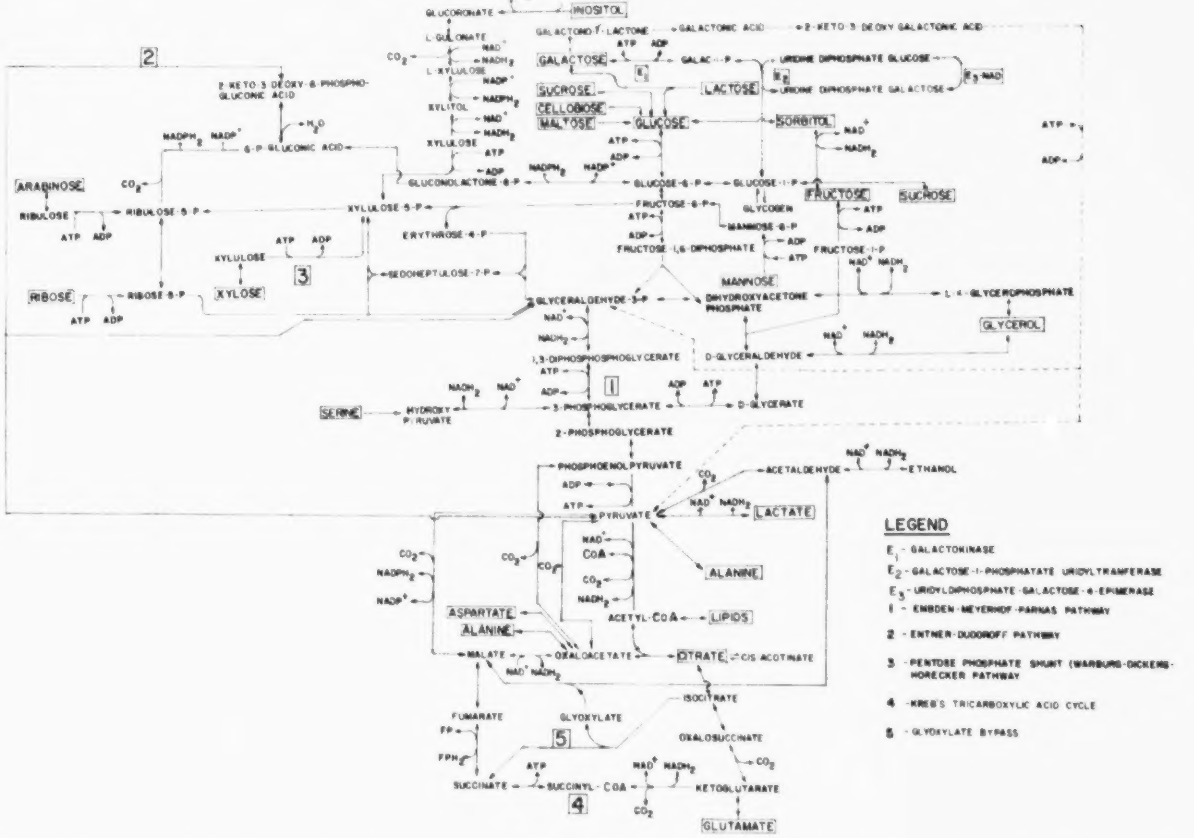

GLUCORONATE
NADPH$_2$
NADP$^+$
INOSITOL
L-GULONATE
L-XYLULOSE
XYLITOL
XYLULOSE
GALACTONO-γ-LACTONE
GALACTONIC ACID
2-KETO-3-DEOXY GALACTONIC ACID
GALACTOSE
GALAC-1-P
URIDINE DIPHOSPHATE GLUCOSE
URIDINE DIPHOSPHATE GALACTOSE
$E_1$
$E_2$
$E_3$-NAD
LACTOSE
SUCROSE
CELLOBIOSE
MALTOSE
GLUCOSE
SORBITOL
2
2-KETO-3-DEOXY-6-PHOSPHO-GLUCONIC ACID
$H_2O$
6-P-GLUCONIC ACID
GLUCONOLACTONE-6-P
GLUCOSE-6-P
GLUCOSE-1-P
FRUCTOSE
SUCROSE
ARABINOSE
RIBULOSE
RIBULOSE-5-P
XYLULOSE-5-P
FRUCTOSE-6-P
GLYCOGEN
MANNOSE-6-P
FRUCTOSE-1,6-DIPHOSPHATE
FRUCTOSE-1-P
ERYTHROSE-4-P
SEDOHEPTULOSE-7-P
XYLOSE
3
RIBOSE
RIBOSE-5-P
MANNOSE
GLYCERALDEHYDE-3-P
DIHYDROXYACETONE PHOSPHATE
L-α-GLYCEROPHOSPHATE
GLYCEROL
D-GLYCERALDEHYDE
1,3-DIPHOSPHOGLYCERATE
1
SERINE
HYDROXY-PYRUVATE
3-PHOSPHOGLYCERATE
D-GLYCERATE
2-PHOSPHOGLYCERATE
PHOSPHOENOLPYRUVATE
ACETALDEHYDE
ETHANOL
PYRUVATE
LACTATE
ALANINE
CoA
ACETYL-CoA
LIPIDS
ASPARTATE
ALANINE
MALATE
OXALOACETATE
CITRATE
CIS ACOTINATE
ISOCITRATE
FUMARATE
GLYOXYLATE
5
OXALOSUCCINATE
FP
FPH$_2$
SUCCINATE
SUCCINYL-CoA
4
KETOGLUTARATE
GLUTAMATE
ATP
ADP
NAD$^+$
NADH$_2$
$CO_2$
LEGEND
$E_1$ - GALACTOKINASE
$E_2$ - GALACTOSE-1-PHOSPHATATE URIDYLTRAMFERASE
$E_3$ - URIDYLDIPHOSPHATE-GALACTOSE-4-EPIMERASE
1 - EMBDEN-MEYERHOF-PARNAS PATHWAY
2 - ENTNER-DUDOROFF PATHWAY
3 - PENTOSE PHOSPHATE SHUNT (WARBURG-DICKENS-HORECKER PATHWAY
4 - KREB'S TRICARBOXYLIC ACID CYCLE
5 - GLYOXYLATE BYPASS

FIGURE 4. INTERRELATIONSHIPS BETWEEN MICROBIAL METABOLISM OF SUGARS, SUGAR ALCOHOLS AND AMINO ACIDS WHICH MAY POTENTIALLY GIVE RISE TO PHASIC GROWTH IN APPROPRIATE COMBINATIONS.
(COMPILED FROM REFERENCES 30, 32, 164, 271 & 300-302)

It appears that the presence of glucose is not a necessary condition for diauxie to occur. Furthermore, the inhibited enzymes are not always *inducible* in nature, for Magasanik (26) has shown that a number of enzymes, e.g., penicillinase, nitrate reductase, titrathionate reductase, most amino acid decarboxylases, etc., all known to be *inducible*, are formed in the presence of glucose. Whether inducibility is a necessary condition or not, it is apparent that this characteristic of the enzymes proved to be a popular criterion for explaining the occurrence of diauxie until answers were sought to the questions as to why certain enzymes should be inducible at all whereas others are constitutive; why the same enzymes appear to be constitutive in one species and inducible in another (e.g., the galactose enzymes, though generally *adaptive*, are found to be *constitutive* or *semi-constitutive* in certain strains of *E. coli* (34)); and why constitutive enzymes have to be always present irrespective of the nature of the substrate. Whereas these questions have not been completely settled, the intriguing phenomenon of diauxie and the accompanying substrate-enzyme interactions have been the subject of intensive research which has unraveled the role of the environment, evolution, and heredity in causing the phenomenon.

## Evolutionary Development of Microbial Response Mechanisms to Environment

In the face of competition and changing environment, microbes have developed certain physiological tricks for survival by rapid multiplication. Confronted with continual change of environment, the hereditary characteristics and the enzymatic activities of microbes have been adjusted to permit maximum efficiency of nutrient assimilation and energy production for syntheses. This situation exists as a result of evolution and the guiding principle of metabolism in a given environment is the achievement of the maximum possible growth rate (143). Development of a large population by virtue of short generation time and the great power of dormancy and sporulation after disappearance of the favorable micro-environment almost guarantees survival (144). The type of physiological adjustment would depend on the duration and physico-chemical nature of environmental variation to which the species is subjected. It has been asserted that environment controls the morphology, content of cellular reserves (e.g., capsular materials, polysaccharides, volutin, and lipid inclusions )(145), transfer of genetic material by transformation, transduction, and conjugation (146), initiation of sporulation and germination (147), genetic control of metabolic functions (25,143), and the enzymic make-up of a cell (144). In the presence of persistent adverse conditions, the microorganisms become increasingly tolerant to toxic substances and may even finally decrease their generation time by undergoing induced mutations.

In the process of proliferation, the cells decrease the intracellular entropy by increasing the entropy of the environment so much so that the system comes to an equilibrium and becomes dead. No further exchange of mass and energy can take place between the cell and the surrounding environment and the micro-environment of the cell becomes unsuitable for growth. Studies of environmental effects on nutrition have often furnished guidelines for provision of optimum environment and fermentation and cell yields have increased 100 fold in some cases. Such studies provide means for engineering manipulation of environmental factors for the purpose of obtaining the desired type of metabolism and maintaining it at the desired level.

## Adaptation and Induction

In the literature, adaptation and induction have been used synonymously by some authors notwithstanding the subtle difference in the connotations of the two terminologies. Adaptation refers to the internal adjustments taking place within an organism to make it better suited for growth and reproduction in a changed environment (148). The term applies to individual organisms or a taxonomically homogeneous or heterogeneous population of organisms. Stanier (144) has defined evolutionary adaptation to constitute moulding of the genotype of an organism through evolutionary history by selection and mutation to fit the mean conditions of long-term, unidirectional and systematic environmental variation. On the other hand, physiological adaptation comes into play when the environmental variations fluctuate randomly and occur constantly over periods that are short in comparison to the life span of the individual organism. This latter type of adaptation, which is important in the interpretation of diauxie, involves direct phenomic accommodation to the change against the background of an existing genome* (144). In other words, physiological adaptation, which has come to be known as adaptation or enzymic adaptation, involves no change at the nuclear (genetic) level but involves the activation of gene-controlled enzymatic processes (25, 144) which were inoperative under previous environmental conditions. It is due to this type of adaptation that the organism has the potential ability to respond rapidly to environmental fluctuations of short durations. A different type of physiological adaption in response to long-term cyclic environmental changes and involving alternate selection of genotypes (144) is not of much importance as far as causation of diauxic phenomena is concerned.

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*Genome refers to a set of chromosomes derived from a zygote or gamete. Genotype refers to the genetic constitution of an individual or to a group of individuals possessing the same genetic constitution. On the other hand, phenotype refers to the character of an organism expressed in response to the environment. Phenotype may also refer to a genotype exhibiting the same phenotypic characters.

Enzymic adaptation simply refers to phenotypic change in enzymic constitution of the organism by dint of de novo synthesis of enzymes in response to a changed environment but unaccompanied by a change in the genotype (149-151). When the change involves enzyme formation (increase in substrate-specific activity and apoenzyme (protein) content (34)) in the presence of a new nutrient or substrate, the phenomenon is referred to as a process of induction. Thus induction is a mode of physiologic adaptation in the event of a change in the nutrient composition of the environment and is associated with nutrient-induced enzyme formation. Adaptability, or the potential capacity to perform the adaptive synthesis of a required enzyme, is clearly defined by the genotype (149-151).

The fundamental characteristics of the induction effect, as have been established by the work of Monod and co-workers (25,34,150,152) with the lactose system of E. Coli and which are now generally accepted, are as follows:

1. Formation of most enzymes attacking exogenous substrates is specifically increased in the presence of specific substrates and no

appreciable enzyme synthesis occurs in the absence of the specific substrate. A low residual level of some enzymes may however be maintained even in the absence of the specific substrate for "subsidiary" purposes (52).

2. Adaptive enzymes are completely specific in activity toward the inducing agent.

3. According to the *generalized induction hypothesis* and the *internal induction model* of Monod, formation of all enzymes has to be stimulated by an inducer. The induction of the so called *contitutive enzymes* is "masked" due to the fact that they are induced by *internal induction*, the *internal inducer* being an intermediate in the metabolism of other substances not detected in the growth medium. Pollock (153) has also proposed a similar model. This concept of internal induction has received experimental support (152,153) and it is believed that enzymes involved in the biosyntheses of essential metabolites like amino acids, coenzymes, etc., must generally behave constitutively (152).

It has been established by various investigators (25,34,153a) that the protein fraction of adaptive enzymes is synthesized *de novo* from amino acids or other rudimentary building blocks and does not derive any significant fraction of its sulfur (154) or carbon (155) from any preexisting protein. These observations therefore rule out the possibility of activation of any existing enzyme, or formation from the breakdown products of existing protein molecules, to yield the adaptive enzymes during induction. The increased yield of the new enzyme, amounting to 1000 to 10,000 fold in terms of units per mg dry weight of cells (25), results from the assimilation of the inducer substrate. The synthesis of enzyme-protein requires chemical energy derived from oxidation of substrate or polysaccharide reserve (156), and de novo synthesis requires the participation of a functional and utilizable energy generating mechanism (157). Thus, adaptation does not occur when washed cells are transferred to a nitrogen-free medium containing the inducer substrate (126) because protein synthesis cannot occur without a nitrogen source being present. Further proofs of de novo synthesis of new enzymes and the associated energy requirement come from experiments in which induction did not occur in a "complete" medium in the presence of DNP (2-4-dinitrophenol)(156-158), arsenate (159), or azide (159,160), all of which prevent the utilization of energy generated by substrate metabolism and thus inhibit synthetic abilities.

Very often the presence of an inducer induces formation not of a single but of several enzymes sequentially involved in its metabolism. Accordingly, Stanier (161) and Pardee (143) have postulated that a substrate may induce an enzyme and is converted by it to a metabolite which in turn induces a second enzyme. The latter enzyme changes the metabolite into another compound which in turn induces the next enzyme in the sequence and so on until a whole series of enzymes in tandem are induced and an entire pathway may come into existence.

Considerable information has been accumulated on the kinetics of enzyme formation. After reviewing the data of various investigators including his own, Monod (34) concluded that enzyme formation follows an autocatalytic function of the form

$$\frac{dX^e}{d\theta} = k_e X^e_\theta X^s_\theta \tag{9}$$

where $X_{\theta}^{e}$ = concentration of induced enzyme at time $\theta$

$X_{\theta}^{s}$ = concentration of inducer substrate at time $\theta$

In a later review by Jacob and Monod (25) it was concluded ". . . that enzyme activity increases at a rate proportional to the increase in total protein in the culture . . .," and that activation and deactivation of the induction follow very rapidly upon the addition or removal of the activator. Knox (162) has shown that induction might be suppressed at high temperatures and stimulated at low temperatures.

It is apparent therefore that, in the growth phases before the onset of the exponential growth, the cells adjust to the new environment by initiating the process of induction and gradually producing the enzymes necessary for metabolism of the available nutrients. It should also be noted that the permease systems are induced only in the presence of specific substrates in a manner completely similar to induction of metabolic enzymes (12,25,34,140,143).

## Economical Operation of Cellular Processes and Diauxic Phenomena

It has been asserted by Pardee (143), Davis (31), and Magasanik (163) that, in the interest of survival by rapid growth, a cell tries to select the most economical metabolic route for synthesizing each constituent of the protoplasm under a given environment with the least investment of enzymes and use of the enzyme making machinery. The energy saved can be channeled to rapid production of cell constituents. This mode of action on the part of a microbial cell has come about as a result of evolutionary adaptation.

The glucose effect or the inhibitory effect of glucose has been explained by the fact that degradation of glucose can be accomplished by at least three routes (164)(see Figure 4) yielding a wide variety of metabolites which can also be produced from other organics such as galactose, lactose, maltose, glycerol, inositol, etc. More importantly, because of the existence of alternate degradative pathways and the capability of organisms for simultaneous use of all of the pathways (32), glucose is generally more rapidly utilized than other carbon sources (163). It has been stated by Magasanik (163) that the intermediary metabolites are formed from glucose in a normal cell at a rate more than sufficient to saturate the capacity of the cell to convert them to the immediate precursors of proteins, fats, glycogen, and nucleic acids. Thus, a cell provided with glucose and an alternate carbon source would obtain the various metabolites by degradation of glucose alone as long as these precursors can be supplied at the rate of their utilization via other pathways. The manufacture of enzyme proteins for catabolism of the alternate carbon sources would only mean avoidable expenditure of energy and imposition of additional burden on the protein making machinery. The preservation of the alternate (optional or secondary) carbon source has the additional advantage in that this source can be utilized at a later stage of growth when the supply of glucose falls to a level that can no longer furnish precursors at rates commensurate with the demand exerted by the synthetic pathways. While the kinetics of production of various key metabolites from glucose and other carbon sources have not been compared on the basis of theoretical analysis (such analysis being difficult because of the unavailability of sufficient data on the thermodynamics and rate constants of individual enzymatic steps), comparison of specific growth rates on glucose and the alternate substrates as sole carbon sources indicates that it is kinetically advantageous to obtain the precursor metabolites from glucose.

The above concept is well accepted and is useful in explaining most of the diauxic effects whether glucose is involved or not. The diauxic effects with glucose and other sugars or sugar alcohols are easily explained by the fact that the degradative paths of all inhibited sugars contain inducible enzymes and join the catabolic pathway for glucose. However, there exist some anomalous reports regarding the role of glucose as the preferred substrate. Hamilton and Dawes (139) have reported that, in media containing glucose and the organic acids of the Krebs cycle, P. aeruginosa utilized the acids in preference to glucose. This is to be expected since, when the intermediates of the glucose catabolism are directly available, it is economical for the cell to use them directly from the media. Moreover, it was shown that enzymes for glucose breakdown by the Etner-Doudoroff pathway were inducible in P. aeruginosa (140). It is therefore not surprising that the cell would profit by not synthesizing the inducible enzymes whose end products are supplied in the medium.

In contrast to the observation of Hamilton and Dawes (139,140), Ravin (129,130) observed preferential utilization of glucose by A. aerogenes during growth in a medium containing glucose and citric or acetic or succinic acid. In this system, the acids are relegated to the role of secondary substrates because A. aerogenes possesses the constitutive series of enzymes of the Embden-Meyerhof-Parnas pathway (163) in addition to the Entner-Dudoroff system of inducible enzymes, whereas the acids serving as exogenous substrates have inducible permeases (165). It becomes more economical for the cell to postpone the manufacture of the inducible permeases and to obtain the acids by breakdown through a constitutive as well as the additional inducible system of glucose enzymes. The apparently contradictory results of Hamilton and Dawes and Ravin point to the importance of considering the physiology of the organism in interpreting or predicting diauxic effects. Prediction of diauxie from consideration of the nutrient composition alone may prove to be disappointing in some cases.

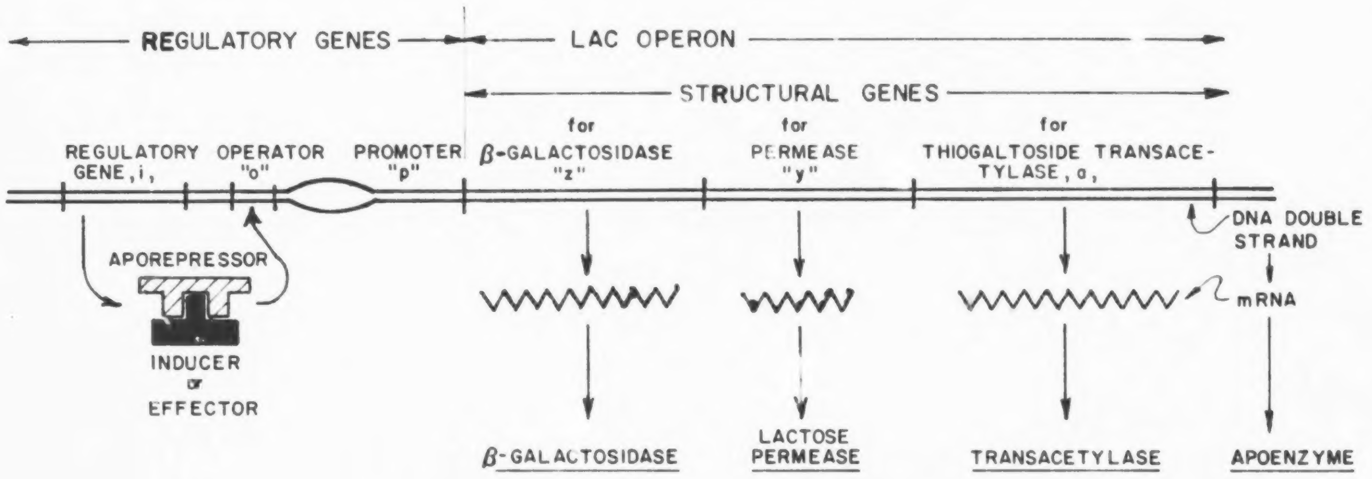
## Mechanisms of Controlled Enzyme Synthesis

A question which arises at this point is that of cellular differentiations during diauxie between the different carbon sources and turning on the appropriate enzyme synthesizing system to produce more enzymes for the preferred substrates, while preventing the formation of other enzymes at the same time. A number of hypotheses have been advanced in clarification of the phenomenon of cellular differentiation of alternate substrates. Magasanik (163) has critically reviewed the limitations of these various hypotheses.

In reporting the discovery of diauxie, Monod (7) had postulated that the phenomenon arose because of the inhibition of induction of the inducible enzymes of the alternate substrate. Since then, much progress has been made in elucidating the mechanism responsible for initiation of enzyme production upon stimulation by an inducer and halting enzyme synthesis when the substrate is absent or is removed from the environment.

An understanding of the mechanism of such controlled production of enzymes was expected to provide clues to the understanding of the mechanism of inhibition of the process of enzyme formation which causes suppression of uptakes of alternate substrates and *diauxie*.

The present theory concerning induced biosynthesis of enzymes is due to the brilliant work of Jacob and Monod (25,166) which won them the Nobel Prize in Medicine in 1965 (167). The Jacob-Monod *Operon* Model of controlled induction may be described as follows. The molecular structure of proteins is determined by the *structural genes* (*z gene*) of the chromosome which act by forming a cytoplasmic *transcript* of themselves in the mRNA (*messenger* or *template* RNA) which in turn participates in protein synthesis on the ribosome. The *structural gene* controls the structure of proteins by dictating the sequence of amino acids in the protein according to its code of bases transcribed on to the mRNA. The synthesis of the mRNA, which in turn is involved in the synthesis of the apoenzyme, is initiated at a certain point or *operator* site, *o* (see Figure 5). The control of the expression of the structural gene is effected by a *repressor* molecule, synthesized by a *regulator gene* (*i gene*), which tends to combine specifically and reversibly with the DNA strand by virtue of the latter's steric structure at the *operator* site, *o*. The combination blocks the initiation of transcription and therefore, formation of the mRNA by the *i genes* which may form part of a system of controlled *structural* genes constituting an *operon*. In the presence of the *inducer* (also called *effector*), the *operator-repressor* complex is destroyed by the *inducer* molecule, thus allowing activation of the *operon*, i.e., initiation of formation of mRNA at the *promoter*


REGULATORY GENES
LAC OPERON
STRUCTURAL GENES
REGULATORY GENE, i,
OPERATOR "o"
PROMOTER "p"
for β-GALACTOSIDASE "z"
for PERMEASE "y"
for THIOGALTOSIDE TRANSACETYLASE, a,
DNA DOUBLE STRAND
APOREPRESSOR
INDUCER or EFFECTOR
mRNA
β-GALACTOSIDASE
LACTOSE PERMEASE
TRANSACETYLASE
APOENZYME

LEGEND:

LAC OPERON - COMPRISED OF GENES FOR CONTROLLING THE SYNTHESIS OF APOENZYMES
"i" - REGULATOR GENE
"o" - OPERATOR (SITE OF INITIATION OF SYNTHESIS OF mRNA)
"p" - PROMOTER SITE
"z" - STRUCTURAL GENES FOR β-GALACTOSIDASE
"y" - STRUCTURAL GENE FOR LACTOSE PERMEASE SYNTHESIS
"a" - STRUCTURAL GENES FOR TRANACETYLASE SYNTHESIS

FIGURE 5. MECHANISMS OF CONTROLLED SYNTHESIS OF ENZYMES. (Example: Mechanisms of control of Expression of the lac operon).

site, p. The mRNAs carrying the codons for apoenzyme formation are unstable and are destroyed in the process of information transfer. The rate of enzyme synthesis is therefore controlled by the rate of mRNA synthesis.

In reviewing the alternate models (168-172) for regulatory systems, Beckwith (173,174) has concluded that the Jacob-Monod model is the simplest and in none of the systems reported on induction is there yet any strong evidence against the operon model. However, the repressor molecule, thought to be a RNA molecule by Jacob and Monod, was shown to be a protein molecule (173,175,176). The validity of the above version of the Operon Model has been proven by the work of several authors (175,177,178).

The essence of the above model is that the control is negative in nature, i.e., the expressions of the genes are repressed by rule and can only be derepressed in the presence of the effector (174) which may also be the inducer or a catabolite of the inducer. As far as enzyme formation is concerned, the presence of the inducer has the effect of positive feedback (31) which involves derepression of the structural genes and continued production of the enzymes; however, the quantity of enzyme formed would be increased indefinitely regardless of the need as the concentration of inducer is increased. As this conclusion is in contradiction to the actual experiences with enzyme synthesis, there must exist other mechanisms for controlling over-production and possible wasteful excretion of the enzymes. The repression effect, which explains the mechanism of control of over-production, also elucidates the mechanism of inhibition of formation and/or activity of the inducible enzymes of the secondary substrate during diauxic growth.

## *Catabolite Repression*

Neidhardt and Magasanik (179,180) have proposed the mechanism of *catabolite repression* as a mechanism for checking over-production of any inducible enzyme. According to this model, the rate of synthesis of a catabolic enzyme will depend on the presence of its specific inducer as well as on the level of the catabolites produced by any catabolic series of enzymes. In catabolism, situations are often encountered where different pathways lead to the same restricted group of catabolites as their ultimate end products. According to Magasanik (163), these catabolites are capable of repressing not only the enzymes of the pathway by which they are formed, but also the enzymes of any catabolic pathway that is potentially capable of producing them. Increased production of catabolites due to high concentration of substrates leads to the accumulation of catabolites and increased repression of the formation of enzymes responsible for production of the catabolites themselves. This *negative feedback* control mechanism, coupled with the *positive feedback*, sets the steady state level of the enzymes in the cell despite variable levels of substrate in the environment (31,163). It is rather difficult to locate the catabolite responsible for the repression. Ames and Martin (181) have suggested that, for any particular system, the *repressor*[*] catabolite is probably that breakdown product of glucose which is also the immediate end product of the reaction catalyzed by the inducible enzyme. The experiments of Neidhardt and Magasanik (138) indicated that different catabolites are responsible for repressing different catabolic enzymes. McFall and Mandelstam (182) suggested that the repressor molecule is in all probability the immediate end product of the enzyme reaction producing the same or some closely related substance. From an analysis of the work of Gorini (183), David (31) reached the conclusion, now regarded as valid, that repression takes effect only after some level of catabolite is reached; therefore, this mechanism is coarse in response and control of enzyme production.

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[*] The term *repressor* was used by Jacob and Monod (25) to designate the *i gene* product which combines with the operator. The repressor of catabolite repression on the other hand is an intermediary product of the catabolic pathway and plays a role in repression of enzyme formation by negative feedback. To avoid confusion, Davis (31) proposed the name *apo-repressor* for the *i gene* product of the Jacob-Monod model.

Nakada and Magasanik (184) indicated that the repressor-catabolite somehow prevents transcription of the structural genes of the inducible enzyme. McFall and Mandelstam (182) theorized that the catabolite repressor is a separate control factor acting at some point of the DNA which does not involve the controlling genes or the structural genes of the Jacob-Monod model. In a more recent exposition, Loomis and Magasanik (185) have discovered a new *CR gene*, analogous to the regulator or *i gene* of induction, which has been postulated to control the synthesis of a macromolecular repressor involved in the control of the structural operons (*z genes*). The repressor (not to be confused with the *i gene* repressor) is stereospecific towards the catabolite and to the appropriate portion of the operon analogous to the *operator* site in the Jacob-Monod model. It is interesting to note that Loomis and Magasanik (185) concluded that the *i gene* product and the *CR gene* product do not appear to block the *z gene* transcription at the same operator site, a conclusion reached earlier by McFall and Mandelstam (182).

### End-Product Repression

End-product repression is analogous to catabolite repression and is another method of negative feedback control, but operational only on biosynthetic enzymes (31,182,186,179). The repression is brought about by the accumulation or the presence of the end product, defined as the last molecule in a biosynthetic pathway before incorporation into a macromolecule (31), which affects only the enzymes responsible for its own biosynthesis.

### Retro-inhibition or Feedback Inhibition

The term first coined by Novick and Szilard (198) involves the inhibition by a catabolite or end product of the activities of existing enzymes whose functioning do not contribute to the economical operation of the cellular processes. Although this mechanism involves negative feedback, it should not be confused with repression which inhibits the synthesis of the enzymes (25,26,31,143,193). It is believed that the cell can rapidly inactivate the existing enzymes in the event that these enzymes are not needed due to the possible operation of a more economical enzyme sequence (31). While repression provides a coarse control for enzyme repression, retro-inhibition endows the cell with an instantaneous or fine control of enzymatic activity (31). Ample evidences have been accumulated (26,31,143,199,200,202) to show that frequently, if not as a rule, the catabolite or the end product competitively inhibits the enzyme responsible for its own formation, and inhibition follows the well established kinetic law of competitive inhibition (27, 29,203).

Figure 6 presents a schematic representation of the means of

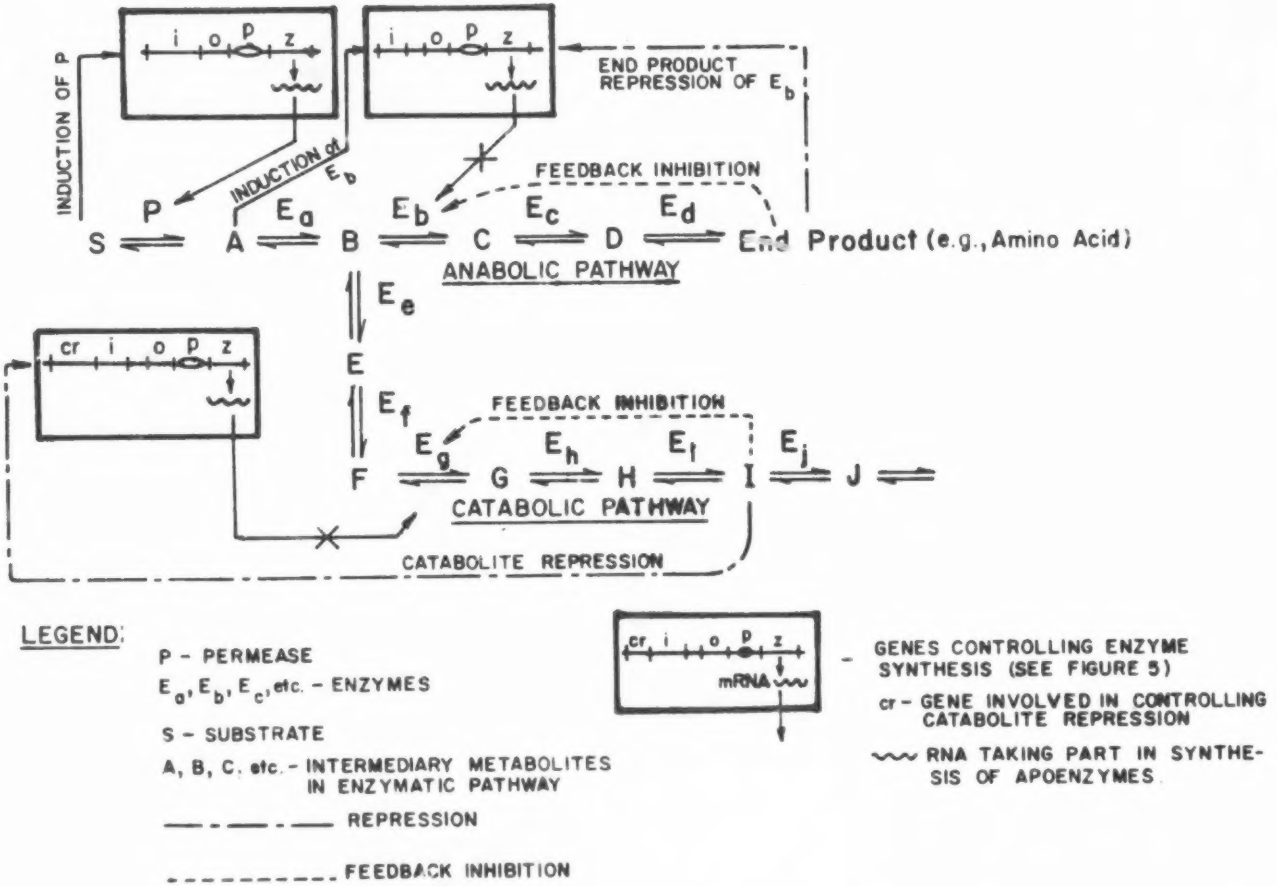
INDUCTION OF P
i o p z
INDUCTION of E_b
END PRODUCT REPRESSION OF E_b
FEEDBACK INHIBITION
S
P
A
E_a
B
E_b
C
E_c
D
E_d
End Product (e.g., Amino Acid)
ANABOLIC PATHWAY
E_e
E
E_f
cr i o p z
FEEDBACK INHIBITION
F
E_g
G
E_h
H
E_I
I
E_j
J
CATABOLIC PATHWAY
CATABOLITE REPRESSION
LEGEND:
P - PERMEASE
E_a, E_b, E_c, etc. - ENZYMES
S - SUBSTRATE
A, B, C, etc. - INTERMEDIARY METABOLITES IN ENZYMATIC PATHWAY
REPRESSION
FEEDBACK INHIBITION
cr i o p z
mRNA
- GENES CONTROLLING ENZYME SYNTHESIS (SEE FIGURE 5)
cr - GENE INVOLVED IN CONTROLLING CATABOLITE REPRESSION
RNA TAKING PART IN SYNTHESIS OF APOENZYMES

FIGURE 6. SCHEMATIC REPRESENTATION SHOWING CONTROL OF SYNTHESIS AND/OR ACTIVITIES OF THE ANABOLIC AND THE CATABOLIC ENZYMES BY INDUCTION, END PRODUCT REPRESSION, CATABOLITE REPRESSION AND FEEDBACK INHIBITION.

controls of synthesis and/or activities of anabolic and catabolic enzymes by end product repression, catabolite repression, and feedback inhibition.

## Role of Regulatory Mechanisms in Controlled Uptake of Competing Substrates

The above mechanisms constituting parts of the total regulatory mechanism of the cell for controlled production of enzyme or any form of protein have been developed as a result of evolution and in the interest of maximum growth by deploying a minimum number of efficient enzymes. The positive and negative feedback are independent control mechanisms (182). They are activated by different regulators (204) which may be the substrate or a structurally related compound (26) acting as inducer for the activation of enzyme syntheses by positive feedback or a catabolite or end product activating the repression mechanism to control its own concentration level as well as the activities of the enzyme producing it. Due to the coexistence and the expression of potential capability of any of the mechanisms or any combination of them (26,31,143, 182), enzymes will not be produced unless the inducer is present; and even when the inducer is present, production of enzyme is controlled by the level of intermediary metabolites or end products. Any undue increase in the flow rate of a metabolic process due to induction is prevented first by inactivation of the enzymes (fine control) by inhibition, and finally by turning off production of the enzymes when the metabolite levels have attained a certain level. Figure 7 shows the various types of feedback mechanisms and their functions. Detailed discussions on regulatory mechanisms may be found in some of the excellent reviews on the subject (174,205-207).

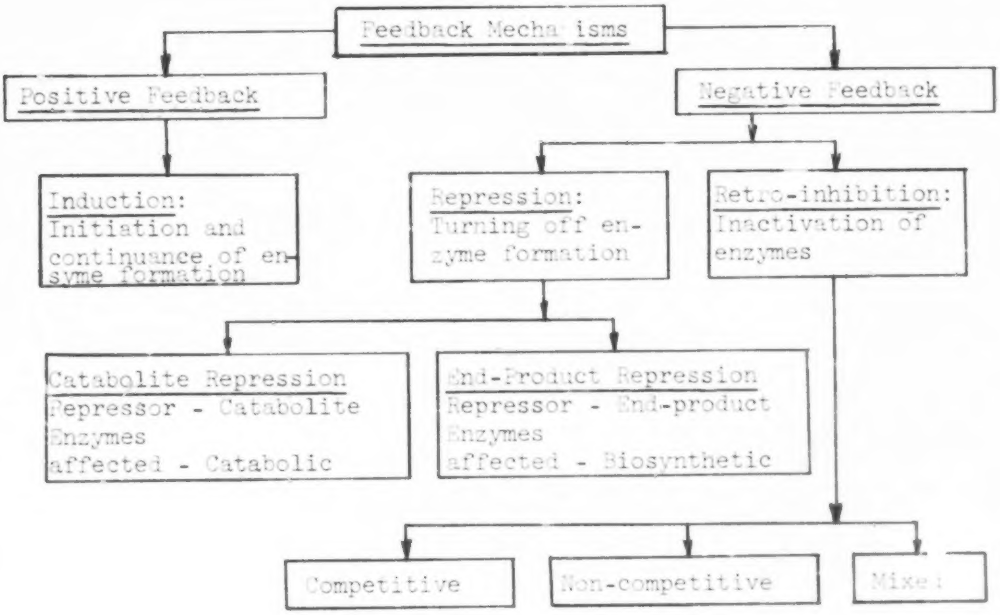
Feedback Mechanisms
Positive Feedback
Negative Feedback
Induction: Initiation and continuance of en-syme formation
Repression: Turning off en-zyme formation
Retro-inhibition: Inactivation of enzymes
Catabolite Repression
Repressor - Catabolite
Enzymes affected - Catabolic
End-Product Repression
Repressor - End-product
Enzymes affected - Biosynthetic
Competitive
Non-competitive
Mixed

Figure 7. Classification of Feedback Mechanisms for Controlled Enzyme Formation

Although a few cases are known (143,208) where genetic selection of cells has given rise to the diauxic phenomenon, by far the majority of the cases can be explained by mechanisms akin to physiological adaptation. As Monod (34) has asserted, diauxie is not due to a differential rate of physical diffusion of nutrients since there is no reason for molecules of nearly identical size and chemical properties (e.g., glucose and galactose) to diffuse at different rates. Diauxie can occur in solid (209) or liquid media. It is not restricted to aerobic environment, since the data of Chynoweth, *et al.* (88) indicated (though not realized by these authors) preferential uptake of glucose by a heterogeneous population of anaerobes. The phenomenon arises due to competition between several carbon and energy sources, leading to severe interaction between the enzyme forming systems which tend to be activated by their respective substrates. Despite the fact that a cell is capable of manufacturing all of the inducible enzymes for different inducers, only the ones capable of aiding most rapid growth are formed while the formation of others is controlled by feedback repression mechanisms. That repression involves inhibition of formation and not activity of enzymes, as has been shown conclusively by some investigators (18[illegible],19[illegible]). When a cell population has been grown in a secondary substrate (Monod's Class B type), inducible enzymes are already existent in the cells and, upon transfer into a medium containing a mixture of Class A and Class B compounds, both feedback repression and feedback inhibition would take effect. The former mechanism would completely block further syntheses of inducible enzymes of Class B inducer, while the latter mechanism would inactivate the existing enzymes of the organisms of the inoculum. However, an important difference in the last case is that growth in the first cycle would be partly attributable to inhibited assimilation of the secondary substrate.

An aspect of diauxic growth which is often overlooked is that the permease system is subject to all the mechanisms of control of the metabolic enzymes due to its inducible nature (2[illegible],31,133,138,143,199,200), and diauxic growth has also resulted due to feedback type repression and/or inhibition of permeases by undefined metabolites (138,199,200).

Information on the kinetics of derepression and induction of inducible enzymes during the diauxic lag, which is akin to the lag in single cycle growth, is very scarce. The rate of enzyme synthesis during diauxic lag does not follow the "auto-catalytic" law suggested for simple lag by Monod (25). Induction of the repressed enzymes during the diauxic lag is characterized by an initial explosive burst of preferential synthesis of these enzymes followed by a sharp decline of the concentration of enzymes per cell which then remain fairly constant (31, 183). It has been suggested that, during the diauxic lag, the enzymic adaptation and preferential enzyme synthesis may occur in the absence of demonstrable growth of the bacterial cell due to marked impairment in total protein synthesis (31,182). All available amino acids may be diverted to apoenzyme syntheses and building blocks for other proteins may not be so available (31).

## Diauxie in Heterogeneous Culture

It is believed that diauxic metabolism and growth in heterogeneous culture was first shown by Hulcher, et al. (211) with soil bacteria which utilized amino acids as carbon sources in preference to cellulose from a complex synthetic medium. Gaudy and his co-workers (211-214) have reported extensively on preferential utilization of glucose by heterogeneous population. These authors concluded that glucose was able to repress the synthesis of the inducible enzymes of the secondary substrates (sorbitol, mellibiose, and lactose). In the case of sorbitol grown cells, some sorbitol utilization at a reduced rate was evident during the first phase of growth; it was concluded that feedback inhibition of the sorbitol enzymes by a glucose catabolite was the causative factor (212). However, in another report these investigators presented results showing no uptake of sorbitol from glucose-sorbitol mixture by "young" sorbitol grown cells which would be expected to possess sorbitol metabolizing enzyme (231). In the latter case, inhibited uptake of sorbitol in the first phase instead of complete repression would be anticipated. The absence of feedback inhibition was attributed to "shock loading" of young cells (231). On the other hand, "old" (by virtue of serial subculturing for 21 days) sorbitol grown cells showed no _diauxie_ when fed with a mixture of glucose and sorbitol. The explanation offered was that an undefined fraction of the cells lost the "suppression mechanism" and constitutivity of the glucose enzymes. In light of the preceding discussions on regulatory mechanisms, it is evident that the "suppression mechanism" would be either catabolite repression or feedback inhibition; both mechanisms are activated by a catabolite occurring after the point of convergence of the catabolic pathways of glucose and sorbitol (see Figure 4). Since the "old cells" used both glucose and sorbitol, the key catabolite capable of activating any of the suppression mechanisms must have been present. Under the circumstances, it is not understood how the suppression mechanism could have been "lost" by the cells. Also, the mere fact that glucose is metabolized from the beginning suggests that constitutivity of the glucose enzymes was not lost as claimed by these authors. If the authors implied the occurrence of spontaneous mutation, it is hard to explain how only a segment of the population underwent mutation for sorbitol uptake while the other segment did not under identical environmental conditions. Furthermore, the studies with "young" and "old" cultures (231) yielded results which were in variance with the results of the earlier studies (211,212) by these same investigators. It should be pointed out that sorbitol-glucose is a poor combination for diauxie since both compounds have nearly the same maximum specific growth rate and, at mixtures containing high concentrations of these substrates, the cell does not gain by discriminating between the two substrates. Whether diauxie would occur or not with such combinations of substrates would be determined by the past history of the cells, the ratio of the concentration of the two substrates, and other factors remaining to be identified. Indeed, Gaudy and his associates did not observe in most of their experiments any pronounced diauxic lag in the curves of oxygen uptake, total substrate COD, or bacterial growth.

In an "attempt to confirm the relevance of Gaudy's findings," Stumm-Zollinger (215-218) conducted experiments which showed that galactose grown cells assimilated galactose during the first phase of growth at a reduced rate whereas glucose grown cells failed to degrade any galactose during preferential utilization of glucose. In light of the previous discussion on the regulatory mechanisms in this chapter, it is not difficult to explain these observations. Upon transfer of galactose grown cells to glucose-galactose media, the cells would not produce any galactose enzymes since they can achieve higher growth rates through utilization of glucose. The glucose catabolites plausibly prevent transcription of the galactose operon by inactivating it through the CR gene product. Induction of the galactose enzymes cannot occur as long as the level of the catabolite repressor does not fall below a certain level. However, as the cells already possess the galactose enzymes by virtue of being cultured in this substrate, some galactose utilization is bound to occur (as shown by Stumm's data), but the glucose catabolite would inhibit these enzymes and the kinetics of galactose uptake would be inhibited according to the level of the catabolite-inhibitor. On the other hand, when glucose grown cells, devoid of galactose enzymes, are presented with glucose and galactose, repression of galactose enzyme formation by glucose catabolites would take effect from the beginning and no galactose assimilation would be expected. Thus, regardless of the past history of the cells, catabolite repression would be operative as long as growth on glucose alone is compatible with the most rapid growth achievable and derepression and induction of galactose enzymes would begin only after the level of glucose catabolites (or glucose itself) has fallen below a certain value.

Stumm's explanation of the phenomenon, i.e., that glucose did not interfere with galactose induction during preferential utilization of the former, does not seem to be tenable. If this conclusion were correct, it would be difficult to explain why galactose grown cells did not synthesize galactose enzymes commensurate with the galactose concentration present as well as why galactose assimilation was so severely impaired. In an earlier report, Stumm (218) showed that galactose grown cells completely rejected galactose in preference to exclusive utilization of glucose. This observation is in contradiction with her later work showing inhibited uptake of galactose under nearly identical experimental conditions (215-217). The results of Hernandez (219) with a glucose-galactose system are also in disagreement with those of Stumm as the former investigator observed simultaneous utilization of glucose and galactose.

Stumm (216) has shown that glucose is not inhibitive to galactose assimilation at lower temperature (7°C) thereby indicating that diauxie is a function of temperature. This observation appears to have been indicated by Knox (162) who concluded that inhibition of adaptation is almost nonexistent at lower temperatures.

In contrast to the results of Gaudy and co-workers (211,212), Gates, et al. (220) observed sequential oxygen uptake, indicated by double sags, by heterogeneous microbial populations fed with glucose-acetate and glucose-lactose as substrate combinations. Inasmuch as it is difficult to explain the disagreement between the results of the two groups of investigators, it has been at least demonstrated that sequential oxygen assimilation is a possibility in natural waters. However, the mere occurrence of oxygen utilization characterized by multiple dips should not be construed to be indicative of diauxie, for such multiple sags were also observed by Marlar (221) in a system similar to that of Gates, et al. but with glucose as the sole carbon and energy source.

The emphatic contention of Gaudy and co-investigators (211-214) and Stumm-Zollinger (215-218) that the first phase of diauxie is characterized by "exclusive" utilization of glucose is neither supported by their data at low concentrations of the substrates nor acceptable conceptually. Growth at the highest possible rate certainly cannot be maintained without drawing upon the preserved secondary substrate after glucose has been depleted beyond a critical concentration. Such clear-cut separation of phases of assimilation of the two substrates is atypical of natural phenomena which are often characterized by graduated changes through transition phases. The works of Chian and Mateles (222), Mateles, et al. (223), Mateles (224), and Yannasch (225,226) have demonstrated that consumption of the secondary substrate is indeed initiated at a finite concentration of the primary substrate and there exists a phase of growth when both substrates are assimilated simultaneously. Chian and Mateles (222) and Mateles, et al. (223) indicated that the "catabolite inhibition" theory of Gaudy, et al. (212) and Stumm (216) does not account for the diauxic phenomenon in all of its aspects.

Aside from the confusions created by the above researchers relative to the probable mechanisms of causation of diauxie in heterogeneous cultures, no consideration whatever was given to interference of the permease of the secondary substrate by the primary substrate as a probable cause of sequential assimilation. Inasmuch as the question concerning the criteria for initiation of assimilation of the second substrate is not settled, no model exists, rational or otherwise, to describe the kinetics of substrate assimilation in the different growth phases and the diauxic lag phase.

It appears that the following information is essential for development of a rational kinetic model for substrate assimilation and growth:

1. The growth rate in the first growth cycle at which assimilation of the secondary substrate is initiated.

2. Rates of assimilation of primary and secondary substrate during the transition phase or diauxic lag.

3. Growth yield during the diauxic lag. This information is necessary to delineate the pattern of growth, if any, during the diauxic lag.

A batch culture technique is unsuitable for collection of information of this kind since the interval between the beginning of uptake of the secondary substrate and complete exhaustion of the primary substrate is very short and adequate sampling is not possible to establish much reliable information. The concentration of the primary substrate becomes very low and cannot be determined accurately from single samples at various selected specific growth rates. The validity of the concentrations determined can also be questioned because of the accumulation of excreted metabolites and the continuous change of environment. However, reliable informations can be obtained by employing the continuous culture technique of Monod (227) and of Novick and Szilard (73,228,229). With this technique, any instantaneous growth condition of the batch growth can be "frozen" and maintained indefinitely at a fixed growth rate under constant environmental conditions. Secreted metabolites cannot accumulate as they are constantly flushed out by the continuous flow of fresh nutrients and substrates of constant chemical composition. As most actual processes are operated on a continuous flow-type basis, information obtained by use of the continuous culture technique would be far more meaningful from an applied viewpoint.

## Continuous Culture Technique

The advantages of the continuous culture technique are that precise manipulation and maintenance of the physical and chemical environment are possible and the cells can be held at steady state and constant physiological conditions for long periods. Thus the culture can be continuously maintained at a maximum growth rate with a maximum rate of product formation. The technique has proved to be a versatile research tool as it allows the investigator to control the environment at will. This continuous culture technique has been used for:

a. study of kinetics of synthesis of DNA, RNA, protein (230-233), and glycogen (234);

b. study of kinetics of respiration (62-65,59,235,236), and $CO_2$ production (59,236);

c. study of energy of maintenance (62-65,59,66,67);

d. study of kinetics of inhibition (80);

e. study of mutagenecity of compounds (74);

f. study of induction of enzymes (74);

g. study of diauxic phenomena (134,135,222,223);

h. study of spontaneous mutation rates (73,74,76,77,237);

i. selection of species (238);

j. simulation of aquatic ecosystems (225,226,239) in order to study the nature of shifts of biotic composition of microbial population, nutritional requirements and microbial intractions;

k. anabolic rates (73,74); and

l. study of kinetics of growth (59,62-65,74,96,110,134,135, 225,226,239,246,241).

Since the discovery of the principles of continuous culture and the apparatus (called Bactogene by Monod (227) and Chemostat by Novick and Szilard (228,73)), there has been a tremendous upsurge of interest in this research technique and several major symposia and literature reviews have been devoted to the subject (242-247).

## Principles of Continuous Culture

If a bacterial suspension is removed from a reactor at a rate equal to the flow rate of fresh incoming nutrients in which the concentrations of all nutrients are in excess (except that of the growth controlling substrate), then the volume of the culture would remain constant and the rate of change of bacterial density is given by (58,74,240):

$$\frac{dX_1^o}{d\theta} = k(X_1^s)X_1^o - DX_1^o \qquad (10)$$

where $X_1^s$ = concentration of growth controlling substrate in the reactor

$k(X_1^s)$ = the specific growth rate constant when substrate concentration is $X_1^s$

$D$ = dilution rate (flow rate/reactor volume) which is the number of complete displacements of reactor volume per unit time

$X_1^o$ = concentration of microorganisms in the reactor

$DX_1^o$ = rate at which organisms are washed out

If $X_1^o$ is small when continuous flow is started at a constant dilution rate of $D < k^m$, $X_1^s$ is high due to higher concentration of substrate in the influent ($X_o^s$) and the growth rate, $k(X_1^s)$, tends to rise. Thus $X_1^o$ initially tends to rise causing $X_1^s$ to fall due to bacterial consumption until the growth rate is balanced by the rate of wash out, $DX_1^o$, and a steady state in bacterial and substrate concentration is reached (58,74,240). The system becomes stable in that small accidental fluctuation from a steady state value will set up opposing physical forces of dilution or biochemical forces of growth which would restore the *status quo*. Under steady state, therefore

$$k(X_1^s) = D \tag{11}$$

and

$$\frac{dX_1^o}{d\theta} = 0 \tag{12}$$

i.e.,

$$X_1^o = \text{constant} \tag{13}$$

and so

$$X_1^s = \text{constant} \tag{14}$$

The continuous flow reactor therefore permits the experimenter to work with a constant bacterial density maintained at a constant growth rate provided that the dilution rate is kept constant and below the maximum specific growth rate. If $D > k^m$, bacteria would be washed out at a rate exceeding their rate of multiplication and eventually all cells in the reactor would be eliminated by wash out.

Continuous culture may be *homogeneous* when the contents are completely mixed such that the concentrations of substrate and cells, as well as the physical environmental factors, are uniform throughout the culture. A reactor is *heterogeneous* if the flow is of ideal "plug" or "piston" flow type (248). In this case, steady state concepts apply to a point in the flow path akin to a point in time in batch operation. The culture may be in *single phase* if grown in liquid media or *multiphase* as in biological processes in packed towers and trickling filters

(249). A system is *closed* when microbial cells are completely retained by recirculation of outgoing cells, or it may be *open* if cells are continuously lost (249). Continuous culture may be multistage when several reactors are operated in series or in a combination of series-parallel feeding (249,250).

A *homogeneous* or *completely mixed single phase single stage* reactor may be operated by discontinuous flow which is actuated when a set culture density is reached. The device, called a *turbidostat* (251,252), is characterized by a fluctuating culture volume and "saw-tooth" type of steady state concentration varying about a mean (59). At low dilution and growth rates, the frequency of discontinuous delivery of medium would be low approaching batch conditions and leading to unstable conditions and insensitive control (59). A turbidostat is preferred at high dilution rates and for species having complex growth requirements (252). The device involves the use of complicated electronic gear and a photocell, the operation of which is often hampered due to slime growth on the reactor walls.

The other homogeneous system is the *chemostat* or *bactogene* in which the bacterial density and the growth rate are controlled by keeping the culture volume and the flow rate constant for operation under a chosen growth rate. The chemostat is cheaper, more convenient, and simpler to operate and is also commercially available (252). It is preferred to a turbidostat particularly for operation at lower dilution rates (59).

### Mathematics of Chemostat Behavior

Assuming growth rate to be described by the Monod equation, it can be shown easily from Equations 3 and 10 that steady state substrate concentration is given by

$$X_1^s = \frac{KD}{k^m - D} \tag{15}$$

The steady state bacterial density maintained in the chemostat is obtained from mass balance as

$$Y^o(X_o^s - X_1^s) = X_1^o \tag{16}$$

where $X_o^s$ = influent substrate concentration

$Y^o$ = observed or apparent yield coefficient

From Equations 15 and 16 it further follows that

$$X_1^o = Y^o\left[X_o^s - \frac{KD}{(k^m - D)}\right] \tag{17}$$

The dilution rate at which washout occurs is attained when there is no growth due to consumption of substrate, i.e., from Equation 16 when

$$X_o^s = X_1^s \tag{18}$$

Designating dilution rate at washout as the critical dilution rate $D_c$ (240), one obtains from Equations 15 and 18

$$D_c = \frac{k^m X_o^s}{K + X_o^s} \tag{19}$$

Equations 15, 17, and 19, which were derived by Monod (227), Novick and Szilard (73,74), and Herbert, et al. (240), involve the following implicit or explicit assumptions:

a. complete mixing and homogeneity of culture,

b. growth controlled by a single nutrient,

c. constant growth yield at all growth rates,

d. pure culture and absence of genetic variants,

e. absence of mixed metabolism,*

f. instantaneous mixing of influent particles,

g. instantaneous conversion of influent substrate by reduction of its concentration from $X_0^s$ to $X_1^s$,

h. constant dilution rate,

i. capability of organisms to adjust to growth rates imposed by virtue of the applied dilution rate, and

j. growth at exponential phase.

While some of the above assumptions are easily satisfied by the experimental conditions, others do not hold in many cases. Slime growth or "wall growth" on solid surfaces renders the culture heterogeneous and two phase (253). It has been shown by many researchers that the yield coefficient is not constant at all growth rates (59,62-65,225,236). Mixed metabolism involving release of volatile acids at high growth rates has been observed in some cases (222,235,242).

There are two schools of thought regarding the instantaneous conversion of substrate and the adjustment of growth rate by the organisms.

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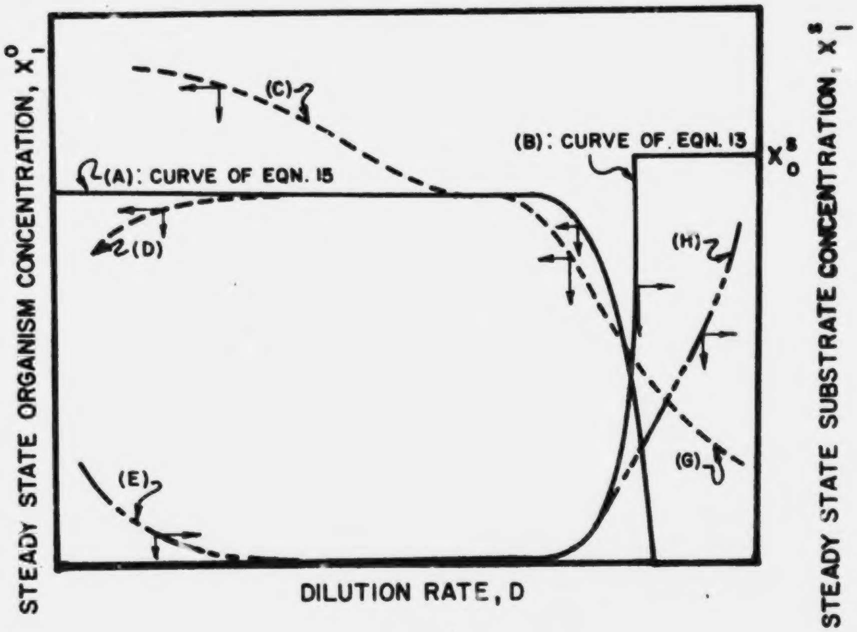
*Mixed metabolism refers to the condition when different pathways leading to different products are operated simultaneously. Thus, under excessive aeration, yeast may produce more cells and $CO_2$ as well as ethanol and $CO_2$ by an alternate pathway (242).

While Harte and Webb (135), Kornberg, et al. (254), Herbert (255), and Martin and Washington (241) have held onto the view that the assumption is valid, Mateles, et al. (256) and Bungay and Bungay (253) have disagreed with this viewpoint. Humphrey (257) has mentioned that there is a definite lag before organisms respond to incoming nutrients. The problems of instability and shift of steady state to new values of substrate or organism concentrations due to spontaneous mutations or contamination by genetic variants have been discussed by Novick (73), Powell (237), and Moser (76,77).

It remains a fact that the assumptions are many and the steady state Equations 15 and 17 seem to apply to idealized conditions hardly achieved in practice. Reported deviations of experimental data from the curves of steady state equations (see Figure 8) were so numerous that Herbert (59) considered the agreements with theory to be only of qualitative nature. Powell (58) stated that the disagreements may be due to the fact that the reactions of a living organism are more complex, and the Monod expression for specific growth rate, k (which forms the basis of the above equations), can be regarded only as giving a rough empirical description of k. Further complications are introduced in reactors with heterogeneous culture due to microbial interactions such as commensalism, mutualism, symbiosis, synergism, predation, antagonism, parasitism, neutralism, etc. (253). Bungay and Bungay (253) have concluded that steady states are necessarily oscillatory due to microbial interactions and wall growth in heterogeneous culture.

As shown in Figure 8, several types of divergences have been reported.



$X_0^s$ = INFLUENT SUBSTRATE CONCENTRATION

CURVE (A): ORGANISM CONCENTRATION FROM EQN. 15

CURVE (B): SUBSTRATE CONCENTRATION FROM EQN. 13

CURVE (C): DEVIATION OF ORGANISM CONCENTRATION IN CASE OF NUTRIENT DEFFICIENCY

CURVE (D): DEVIATION OF ORGANISM CONCENTRATION DUE VARIATION OF OBSERVED GROWTH YIELD WITH VARIATION OF SPECIFIC GROWTH RATE.

CURVE (E): DEVIATION OF SUBSTRATE CONCENTRATION DUE TO INHIBITORY EFFECT AT HIGH POPULTION DENSITY

CURVE (G): DEVIATION OF ORGANISM CONCENTRATION ATTRIBUTED TO "IMPERFECT MIXING" AND WALL-GROWTH

CURVE (H): DEVIATION OF SUBSTRATE CONCENTRATION ATTRIBUTED TO "IMPERFECT" MIXING AND WALL-GROWTH

DEVIATIONS REFER TO THE DEVIATIONS OF EXPERIMENTAL OBSERVATIONS FROM IDEALIZED CURVES DESCRIBED BY EQUATIONS 15 & 13.

FIGURE 8. STEADY STATE CONCENTRATION OF ORGANISMS AND THE GROWTH CONTROLLING SUBSTRATE AS FUNCTIONS OF THE DILUTION RATE IN A CHEMOSTAT.

1. Deviations of types (g) and (h) are fairly common and have been attributed to "imperfect mixing" and "wall growth" (58,59). As will be discussed in Chapters VII and VIII, such deviations may also be only due to slime growth. Contois (79) developed an equation for $k(X_1^S)$ to account for deviation on account of wall growth. It is doubtful that deviations of type (h) at higher dilution rates can be attributed to short circuiting and imperfect mixing since the theoretical and experimental analysis of Grieves, et al. (258) and Milbury and Pipes (259) indicated that for a given dilution rate substrate concentration was higher than predicted from theory for a given dilution rate.

2. Divergences of type (c) are observed in the presence of nutrient difficiencies when substrates are diverted for synthesis of cellular reserve materials like glycogen (59,234,58).

3. Deviations of type (c) have been observed by Mor and Fiechter (236) and Jannasch (225,226) in heterogeneous culture and were attributed to an inhibitory effect at high population density.

4. Divergences of type (d) have been frequent (58-60, 62-68, 80,236) and are attributed to variation of yield with growth rate. Yield coefficients are generally observed to be lower at lower growth rates (225). Herbert (59,260) postulated that decreased yield at low growth rates is due to the fact that, in addition to anabolism, cells also have a constant endogenous metabolism.

Endogenous metabolism has been interpreted differently. According to Herbert (59), cell substances are oxidized to $CO_2$ for deriving energy for cellular functions other than synthesis of macromolecules. This concept led to the idea of negative growth. However, Marr, et al.

(66) have stated that the lower yield is not due to destruction of any preformed biomass but rather because a significant portion of the carbon source is diverted to processes that do not result in increase of biomass concentration. Herbert (59) had assumed endogenous metabolism to be independent of growth rate, whereas Button and Garver (261) had evidence indicating endogenous metabolism to be prevalent at lower growth rates. In order for Equation 17 to conform to deviation of type (d) due to variation of yield coefficients, Herbert adjusted the equation of exponential growth as follows:

$$\frac{dX^o}{d\theta} = \left[k(X^s) - k^e\right]X^o \qquad (20)$$

In Equation 20 $\underline{k^e}$ has been defined as the units of equivalent biomass destroyed (that could theoretically be produced with the amount of energy source used for maintenance) per unit of biomass present per unit time (59,80).

Herbert's advocation of endogenous metabolism at all growth rates is contrary to the reports of many investigators (40-42) who have maintained that such metabolism at the expense of cellular reserves occurs only under conditions of near starvation. The suggestion of destruction of preformed macromolecules for derivation of energy, even in the presence of energy substrate, is against the principles of cell economy. It appears that it is more profitable for the cells to derive energy directly from catabolism of the carbon source rather than synthesizing cellular constituents and then catabolizing them. The introduction of the term, $-k^eX^o$, which implies simultaneous synthesis of protoplasm at a rate $\underline{k}$ and destruction at a rate $\underline{k}^e$, is of doubtful conceptual validity notwithstanding the justification from the viewpoint of mathematical description of the deviation of type (d) in Figure 8.

Pirt (68) has pointed out that this concept of negative growth introduced by Herbert (59,260) to account for energy of maintenance is artificial and indirect. Following the concept Duclaux (262), Monod (7), and others (62-65,66), Pirt (68) have defined maintenance energy requirements in terms of energy substrate consumed per unit mass of organism per unit time. It is said that *energy of maintenance* is required by a cell in the form of ATP at all growth rates for motility, active transport of nutrients across the cell wall, hydrolysis of proteins and nucleic acids to their constituent monomers and resynthesis of other micromolecules from monomers, transport of metabolites from one part of the protoplasm to another, cell division, tactic response, and other functions yet to be clearly defined (40-42,53,80). In other words, *energy of maintenance* refers to energy consumed for functions other than production of new cell materials (68). Thus, overall substrate utilization rates may be considered as the sum of the rates of substrate utilization for maintenance and for growth. That is

$$\frac{dX^s}{d\theta} = \left[\frac{dX^s}{d\theta}\right]_{\text{maintenance}} + \left[\frac{dX^s}{d\theta}\right]_{\text{growth}} \qquad (21)$$

Further,

$$-\left[\frac{dX^s}{d\theta}\right]_{\text{maintenance}} = mX^o \qquad (22)$$

In Equation 22 m is the maintenance coefficient in terms of energy substrate consumed per unit mass of organism per unit time, and is considered a constant independent of growth rates.

van Uden (80) has opined that the maintenance requirement is lower at high dilution rates during unrestricted growth. Pirt (68) and Schulze (63-65) defined a term, Y, as the yield coefficient corrected for endogenous metabolism and given by the equation

$$\left[\frac{dX^s}{d\theta}\right]_{growth} = -\,k(X^s)\left[\frac{X^o}{Y}\right] \qquad (23)$$

Overall substrate consumption rate is now given by

$$\left[\frac{dX^s}{d\theta}\right] = -\,k(X^s)\left[\frac{X^o}{Y^o}\right] \qquad (24)$$

where $Y^o$ is the observed or apparent yield coefficient.

From Equations 21, 22, 23, and 24 it is readily seen that

$$\frac{1}{Y^o} = \frac{m}{k(X^s)} + \frac{1}{Y} \qquad (25)$$

where $Y$ = "true" yield coefficient.

Pirt modified Equation 17 of Herbert by substituting the expression for $Y^o$ from Equation 25.

Despite the conceptual differences in accounting for the decreased yield at lower growth rate, the equations of Herbert or Pirt only serve to conform the theory to the observed deviations of type (d) in Figure 8. However, there are reports of yield factors decreasing with an increase in growth rate (58), and also as being maximum at a particular dilution rate above and below which it decreases (236,257). The adjusted equations of Herbert and Pirt obviously would not describe yield variation of the latter types, nor are there any models which would describe deviations of types other than type (d). The evaluation of the steady state equations by Herbert (59) and Powell (58) as being able to conform only "qualitatively" to actual observations seems to be appropriate.

## Population Dynamics and Selection in Continuous Culture

In natural ecosystems, growth of a heterogeneous population in an environment following inoculation with nutrients leads to a change in the environment which is conducive to the growth of a second group of organisms and further environmental change. Over a period of time the succession of various dominant populations is observed starting from bacterial and algal population of the lower trophic levels and higher productivity (by virtue of rapid growth and efficiency) to a population of progressively higher forms of life of higher trophic levels. The phenomena have been described by Bartsch and Ingram (263) and at length more recently by Brock (264).

In a continuous flow reactor, the environment is kept constant and only those species which are endowed with the genetic and physiological adaptive capacities to adjust to this controlled environment will persist. The experimenter can exert selective pressures at will by manipulating the chemical or physical factors of the environment. The survival and proliferation of a species confronted with increases in selective pressure depend on the genetic and adaptive potential of the species as expressed by continued growth in the modified environment. Bryson (238,252) applied this principle for isolation of bacterial variants.

Since the dilution rate determines the mean growth rate, selection of variants may be effected by simply changing the hydraulic displacement rate while keeping other factors constant. The fate of a population is determined by the rate of washout such that if the latter exceeds a certain value (minimum generation time), the population eventually will be removed completely from the reactor (237,74,58,31). In pure cultures, mutants with higher growth rates than parents, called prevalent mutants, ultimately displace the parent cells (58,74) and disrupt the steady state, shifting it to a new position (76,77). Mathematical treatment of the dynamics of mutant populations is presented by Powell (237), Moser (76,77), and Renneboog (265).

Population dynamics or the selection of species by selection of dilution rates has been observed in heterogeneous cultures by many investigators (61,96,99,222,224-226, 239,259,266-270). Survival of a species in heterogeneous culture depends on a variety of less well defined factors, many of them not related to the elimination of the unsuccessful competitor by dilution which itself is only one of the important factors. Though the population remains heterogeneous in nature at all dilution rates due to mutual interactions (e.g., predation, symbiosis, neutralisms, etc.), the relative proportion of each species changes from one dilution rate to another. Thus in lactate and glucose-lactate media, Pseudomonas was found to dominate at high dilution rates (222,239). In the anaerobic environment, Andrews and Pearson (96) observed acid-formers to prevail over methane bacteria at detention times of 2.4 days or less. Due to the mutual interactions, the population of each species is oscillatory at a given dilution rate (253,269). Such oscillations probably lead to the oscillations of the total steady state bacterial density (253,270). The studies of Cassell, *et al*. (270), Collard and Gossling (268), Dias and Bhat (266), Jannasch (239,225,226), Chain and Mateles (222), Mateles (224), and Andrews and Pearson (96) indicate that population change overs are rare during operation at high and low dilution rates and minimum at highest dilution rates. A good rule to follow is that a species is selected against and may be displaced by another species which can grow more rapidly at the low concentrations of the controlling growth factor which occurs in the reactor (74). The principle of selection from heterogeneous culture has been used in the waste treatment plant at Pomona to develop an active population of *Nitrosomonas* for producing nitrified effluent to be subsequently denitrified in a tertiary treatment plant (99).

# CHAPTER III

# FORMULATION OF THE PROPOSED RESEARCH METHODOLOGY

## Introduction

The metabolic characteristics of microorganisms as revealed in the end results of a biological process are the reflections of the mode of operation of a complex network of interdependent catalyzed reactions. Through evolution, the microorganisms have acquired the properties of versatile behavior, only some of which are expressed in the interest of survival by rapid growth in a given environment. Upon stimulation of the genes by the physical and chemical factors of a given environment, the microbial cell produces those enzymes which form the most economical route for processing of nutrients and yielding the maximum growth rate. Economical design and operation of biological processes demand a thorough understanding of the modes of responses to environmental change as well as of the kinetics of catabolic and anabolic processes.

A theoretical development of rate laws for substrate assimilation or biomass formation has not been possible due to the complex and interdependent nature of numerous reactions, some of which are not fully elucidated and/or studied for delineation of their thermodynamic properties. An approach favored by many in preference to pure empiricism is to regard total growth as a rough expression of the total catalytic activity of the network of enzyme systems. The kinetics of growth are then related to the kinetics of substrate utilization or product formation. Of the various empirical, semi-empirical, and theoretical equations advanced for describing simple sigmoid growth curves, the Monod expression, having a sound base on the Michalis-Menten enzyme kinetic equation, has found wide application in pure and mixed culture.

In natural or modulated ecosystems, the chemical and physical composition of the biotic environment is far more complex than the laboratory enrichment media. Two or more nutrients may be potentially growth limiting factors and may compete to be the indispensable nutrient source thus provoking severe interactions between the respective enzyme forming machineries. By virtue of the evolutionary capabilities vested in genes, the organisms solve this dilemma by imploring the enzyme forming system of that substrate which is capable of sustaining the maximum growth rate. Control of production of the enzymes of the less preferred substrate, in the face of persistent stimulation by it, is effected by the metabolites of the preferred substrate. These act either by themselves to quickly inhibit any existing enzymes of the secondary substrate or by inhibiting the transcription of the structural genes of the enzymes of the spared substrate. Sparing of substrates and the sequential assimilation of the substrates finds expression in phasic or multi-cycle growth. In a typical cycle, there is exclusive utilization of a preferred substrate accompanied by repression or inhibition of the enzymes of the less preferred substrate up to a point following which there is derepression and induction of new enzymes and simultaneous utilization of both substrates. There is considerable evidence that the enzymes of the secondary substrate are synthesized de novo at more than normal rate of synthesis in order that normal levels of these enzymes are established quickly. The demands for energy and protein building blocks during the explosive synthesis of these enzymes may cause proportionately more of the assimilated substrate to be diverted to meet this demand, thus sacrificing growth for a short period of time during the transition period (or diauxic lag) between the cycles of growth.

Much research has been done to demonstrate the widespread occurrence of diauxic phenomena in various environments and in pure and heterogeneous cultures. The impacts of the phenomena in controlled biological processes and aquatic ecosystems have also been appropriately emphasized. Information on kinetics of growth and substrate utilization is scarce since the earlier investigators resorted to batch cultures in which growth rates were affected by accumulated metabolic products and accompanying environmental changes and the diauxic lag occupied too short an interval for convenient study. More recently a few researchers have used continuous culture techniques for study of diauxie, since this technique enabled the experimenter to hold the culture at a selected growth rate and under constant environmental conditions for any desired length of time.

The continuous culture theory as developed for growth controlled by a single nutrient has not been sufficient to describe even many pure culture systems for various reasons. However, qualitative agreements between theory and observations can be expected. Heterogeneity of the microbial culture introduces further complications. A shift in population composition ensues in response to any change of environment effected through the shifting of hydraulic displacement rates. The population composition at extreme dilution rates may be significantly different from others and may be described by different Monod type equations as well. The assumption in the development of continuous culture theory has been that the populations thriving at all dilution rates obey the same equation for specific growth rates. This assumption may not be tenable in view of the observations of population dynamics.

A number of questions concerning diauxic metabolism and growth in heterogeneous cultures remain to be settled. These can be enumerated as follows.

1. Under what environmental conditions can the occurrence of substrate interactions and phasic growth be expected?

2. Are the growth constants for individual substrates indicative of its role as the preferred substrate?

3. Would repression and inhibition of the permease system lead to diauxic phenomena?

4. What determines the initiation of induction of the enzymes of the spared substrate?

5. What is the effect, if any, of the secondary substrate on the kinetics of utilization of the primary substrate and *vice versa* when compared to the kinetics of utilization of each substrate as the sole substrate?

6. What are the kinetics of growth and substrate utilization during the diauxic lag? What is the growth yield during this period?

It seems that the first question is answered if the studies by numerous researchers on the response patterns and regulatory mechanisms of microorganisms confronted with environmental change are considered. However, it appears that answers to the last five questions are of basic importance to the understanding of the kinetics of sequential substrate utilization and growth. The experimental technique used for collection of the required information would have to be such that growth yields and utilization of individual substrates could be studied at a number of selected growth rates first in the absence of the interacting substrate and then in its presence to realistically determine the effect of the interaction.

## Design of Experiment

### Selection of the Experimental Technique

A batch technique is unsuitable because the environment progressively deteriorates as the end of the first growth cycle is approached to the extent that the kinetics of growth and substrate utilization may be severely inhibited and no meaningful information on this aspect would be derived. Furthermore, the first substrate disappears at a maximum rate and within a short period of time prior to the diauxic lag phase making it almost impossible to ascertain, with any acceptable degree of reliability, the point of initiation of simultaneous substrate utilization; the cell yield during this period of time would be affected due to the accumulated metabolic products. Since the study of kinetics of diauxie is in effect the study of concentration effects of the preferred substrate on repression and assimilation of the alternate substrate, it was decided that such studies could best be done in a chemostat-type continuous reactor where concentrations and growth rates can be held constant as long as desired, and the interference from excreted metabolites is eliminated.

## Choice of Substrates

The substrates for diauxie should be based upon the following considerations.

1. Substrates should give rise to diauxie when mixed.

2. The enzyme sequence including the permeases should preferably be known for each substrate.

3. Availability of information on inducible and constitutive nature of the enzymes.

4. Availability of thermodynamic data for the enzymatic steps.

5. Availability of information on mechanisms for regulation of enzyme synthesis.

6. Availability of convenient analytical techniques for measurement of concentrations.

Glucose is an obvious choice because of its role in the "glucose effect" and because its biochemical properties have been studied more than those of any other substrate. Similarly, galactose has been used extensively over the past two decades by biologists and chemists in studies of induction, diauxie, genetic control of enzyme formation, and the permease system. The nature of the permease system and the inducible enzymes for galactose metabolism have been elucidated by a number of investigators in recent years. Galactose was, therefore, chosen as the competing substrate for the glucose system.

Metabolism of Glucose. A vast body of information has been accumulated regarding the various enzymatic mechanisms involved in the microbial metabolism of glucose. Since this body of knowledge has been compiled and is well documented, it was deemed unnecessary to include it here.

Metabolism of Galactose. Although the mechanisms of galactose metabolism in mammals have been known for a long time, the enzymes of galactose degradation in microorganisms have been discovered only in the last two decades. An excellent review of the microbial metabolism of galactose has been presented by Kalckar (271). Galactose is taken up by the cell from the medium by a sterospecific permease system (19-21,24,271-277). Each galactose molecule is bound to a stereospecific carrier protein for mediated transport against a concentration gradient (24,277). The galactose-protein complex is reversible and the protein is also able to bind glucose (24). In some strains of *E. coli*, galactose may be transported through the glucose permease (14,24) indicating that the glucose and galactose permease systems can be used interchangeably by some microorganisms. The transported galactose is thereupon acted on by a series of three enzymes of the Leloir pathway: galactokinase, galactose-1-phosphate uridyl transferase, and uridine diphosphogalactose-4-epimerase (also known as galactowaldenase). These enzymes have been isolated in various species of bacteria, yeast, etc. by numerour investigators (278-302) and converted galactose to glucose-1-phosphate, an intermediate in the metabolism of glucose. Thermodynamic constants of the permease system and of the reactions of the three enzymes have been determined by various authors (21,274,301-306). The Leloir pathway is not universally applicable for DeLey and Doudoroff (307) have shown that *Pseudomonas saccharophila* metabolized glucose to pyruvic acid and D-glyceraldehyde-3-phosphate *via* D-galactono-γ-lactone, D-galactonic acid, and 2-keto-3-deoxy-D-galactonic acid. The *structural genes* for the three galactose enzymes form a cluster to constitute the so called *galactose operon* (chromosomal gal segment) which has a common *operator* and is controlled by the same *regulator gene* (308,309). It has been postulated that the *structural gene* of galactose permease is also controlled by the *regulator gene* of the galactose enzymes (308). The galactose permease and galactose enzymes are induced by galactose and D-fucose (6-deoxy-galactose) (309). All three of the galactose enzymes are induced simultaneously (309) and, in the presence of galactose, the activities of these enzymes rise 10-15 fold (273,309). Kepes (19,277) and Horecker, *et al*. (21) concluded that galactose does not exit from the cell by passive diffusion but by mediation with a stereospecific *transporter* protein which is induced in the presence of galactose. Exit activity is enhanced in the absence of the inducer by an unexplained mechanism (21). In the presence of galactose, exit rates of glucose decrease several fold (21).

Buttin (309) has stated that the inhibitor of induction of one of the galactose enzymes may be effective in inhibiting the induction of the permease and the other two enzymes. Since this author has observed inhibition of galactokinase by glucose, it may be concluded that glucose may also inhibit induction of the other galactose enzymes and the permease.

A unique feature of the galactose enzyme system is that the basal levels of the enzymes are rather high in the absence of the inducer (272, 273). This phenomenon has been attributed to *endogenous* or *internal* induction by galactose or UDP-galactose produced from UDP-glucose (272, 273,281,286,287).

The relationship between the galactose and the glucose pathways is shown in Figure 4.

## Experimental Runs

In order to delineate the extent of substrate interactions and concentration effects of glucose at different growth rates, it was decided to conduct three series of experiments in a continuous flow reactor; each series would be comprised of several steady state runs at several selected dilution rates. Glucose and galactose were chosen as the sole carbon and energy source and the growth controlling substrate in the first and second series, respectively. The third series would consist of experimental runs with glucose and galactose as potential growth controlling substrates.

# CHAPTER IV

# KINETIC CONSIDERATIONS

## Development of Enzyme and Growth Kinetics

The growth of a microbial population is determined by the rate of cell division which is exponential in nature in the case of unicellular organisms. The rate of cell division would obviously depend on how fast protoplasmic macromolecules are produced from the nutrients in the given environmental conditions by the previously discussed complex network of enzymatic reactions. It seems, therefore, that the coefficient of population growth rate and variations of it with growth, as well as the ceiling value of the total population, are ultimately dependent on the kinetic constants of the enzymatic reactions. From considerations of the kinetic laws of enzyme reactions and the mode of regulations of the same, it is possible to arrive at a mathematical expression for the variation of specific growth rate of microbial population, and it is not necessary to resort to an empirical equation (e.g., the "logistic" function) whose constants do not seem to have any biochemical significance or orientation.

### Kinetics of Single Enzymatic Reaction

From the mechanism of enzymatic reaction (Equations 26 and 26A) proposed by Brown (101), Henri (102) derived the following equation (Equation 27) for the velocity of the reaction by applying the concept of the law of mass action, which is applicable mainly to a thermodynamically closed system:

$$\text{Enzyme(E)} + \text{Substrate(S)} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{Enzyme-Substrate Complex(ES)} \xrightarrow{k_3} \text{Products(P)} + \text{Enzyme(E)} \quad (26)$$

or

$$E + S \rightleftarrows ES \rightarrow E + P \quad (26A)$$

$$V = \frac{k_3 X_o^E X_\theta^{sE}}{K + X_\theta^{sE}} \quad (27)$$

where $V = \frac{dX_\theta^{sE}}{d\theta}$ (28)

$k_3$ = constant

$X_\theta^{sE}$ = concentration of substrate of the enzyme at time $\theta$

$K$ = constant

$X_o^E$ = initial concentration of enzyme, E

Brown's equation was given sound experimental validation by Michaeles and Menten (103) who presented a slightly different version of Equation 27 as follows:

$$V = \frac{V^m X_\theta^{sE}}{K_m + X_\theta^{sE}} \quad (29)$$

It is to be noted that, when all enzymes are complexed, the velocity, V, would be maximum, $V^m$, so that

$$k_3 X_o^E = V^m \quad (30)$$

In Equation 29, $K_m$ is the Michaelis constant for the substrate. It was shown by Michaelis and Menten (103) that $K_m = (k_{-1}+k_3)/k_1$. Equation 29 was derived with the assumption that the enzyme substrate complex remains in true equilibrium with the enzyme and substrate, which is contrary to the condition of dynamic equilibrium that exists in an open system such as that of a cell. However, application of the concept of dynamic equilibrium by Briggs and Haldane (104) led to a rate expression identical to Equation 29. It should be mentioned that, because of the mathematical similarity between Equation 3 and Equation 29, many writers have inappropriately taken the Michaelis-Menten equation to be descriptive of growth which is not valid *a priori*. The Michaelis-Menten equation is simply the rate expression for a single and isolated enzymatic reaction. For reasons stated in Chapter II, the problem of modeling the behavior of a network of more than 2000 enzymes in a cell is overwhelming and the prospects of arriving at an expression for the overall rate of the system from such an analysis is very dim. However, some progress can be made if some simplifying and aggregating assumptions are made. The model built with the assumption would be acceptable only if it receives experimental support with regard to its closeness to reality and its capability of simulation of prototype systems.

### Kinetics of Multi-enzyme Systems

In a metabolic system having a series of reactions as shown in Figure 9, the rate of the initial reaction is evidently dependent on the nutrient concentration provided in the medium; the subsequent steps in the pathway depend upon the preceding reaction for their substrate. When the system of enzymes has settled down to a steady state defined by a

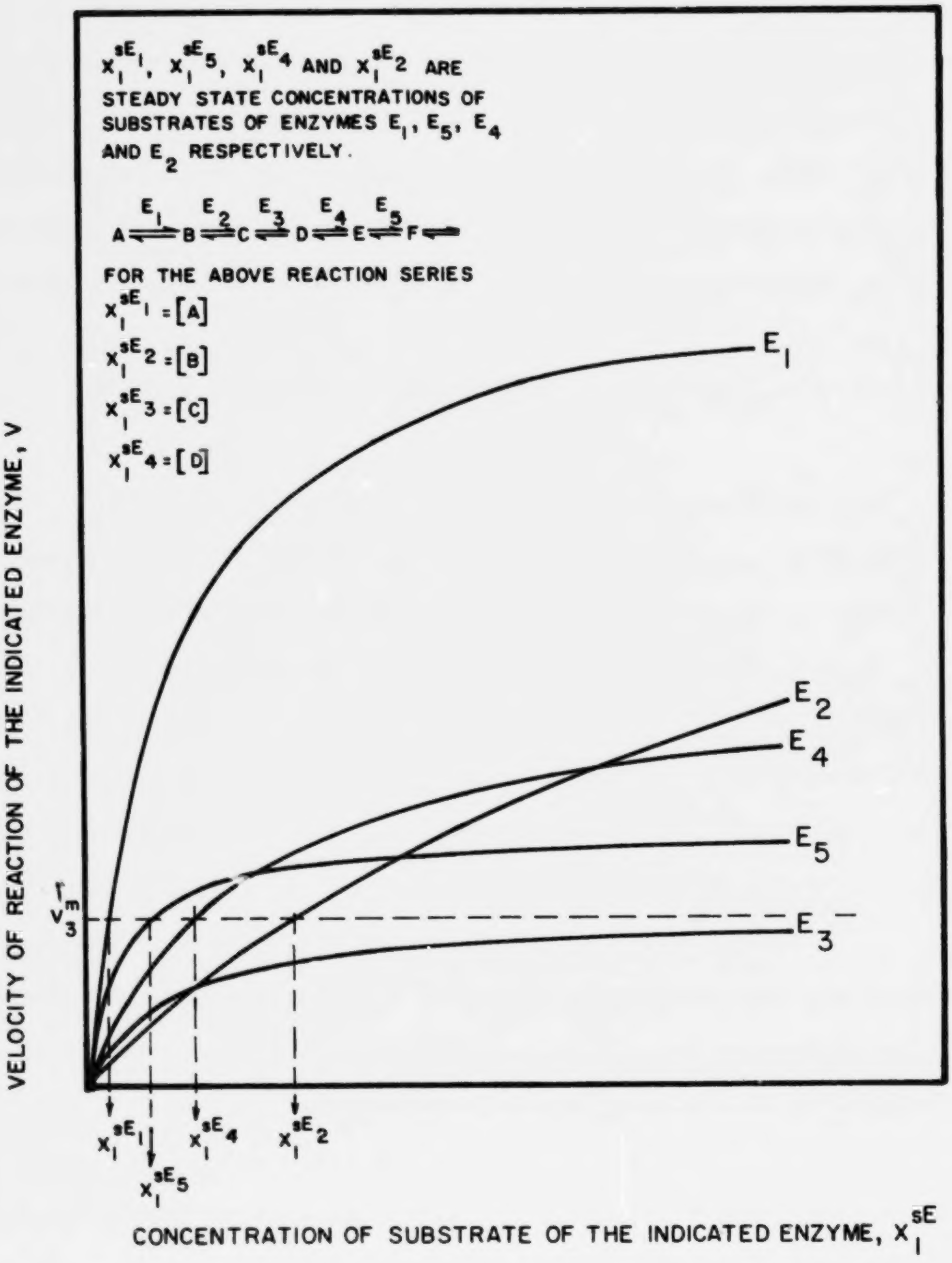


FIGURE 9. MICHAELIS CURVES OF THE COMPONENT ENZYMES OF AN ENZYME SEQUENCE SHOWING THE LIMITING STEADY STATE CONDITIONS.

particular growth rate, the concentrations of the intermediates are small and remain constant to correspond to the total flow of metabolites. The result is that, at steady state, all the reactions must proceed at the same rate. Dixon and Webb (29) and Hearon (310) have concluded that the velocity of a metabolic line is determined by the slowest enzymatic step in the sequence. In a series of enzymatic steps such as follows:

$$A \xleftrightarrow{E_1} B \xleftrightarrow{E_2} C \xleftrightarrow{E_3} D \xleftrightarrow{E_4} \ldots,$$

if for any given environment enzyme $E_3$ is slower than $E_1$ and $E_2$, then Intermediate C would accumulate. Accumulation and increase of concentration of C would tend to accelerate conversion of C to D according to the Michaelis-Menten equation and decelerate $E_2$ by increasing its rate of back reaction. This leads to a process of equalization of velocities, a conclusion which is compatible from the viewpoint of cellular regulatory mechanisms. The rise in concentration of Intermediate C would bring into action the negative feedback mechanisms of retro-inhibition and/or catabolite repression to control the velocity of $E_1$ or $E_2$ or both by inhibiting their activity and/or adjusting the intracellular concentrations of these enzymes. Thus, a key enzyme of the sequence would set the pace for the sequence, and the other enzymes would merely keep up with it. This is the pacemaker concept of Krebs and Kornberg, 1957, (311). Hearon (310) earlier arrived at the same conclusion and further pointed out that the environment affects the velocity of a metabolic route only indirectly to the extent that it determines the slowest enzyme of the sequence. Thus the key enzymatic step may shift from one position in the enzyme system to another with a shift in the environmental makeup.

The different enzymes of the network will have different substrate concentration curves as described by the Michaelis-Menten equation and depending on $K_m$, $V^m$ and the amount of each enzyme present as illustrated in Figure 9. The heights of the curves depend on the concentration of the particular enzyme, which of course can be changed with a change of environment. For any given environment, the enzyme with the lowest $V^m$ will become the limiting or pacemaker enzyme, and its maximum velocity will become the velocity of all other enzyme reactions in the chain. Thus, for the enzyme system represented in Figure 9, no matter how much the substrate concentration may be increased, the velocity of the metabolic line cannot rise above the maximum velocity of enzyme $E_3$. As a consequence, the substrate concentration of each enzyme will adjust itself to that point on its Michaelis curve which gives this rate.

Burton (312) and Dean and Hinshelwood (52) pointed out that the concept of pacemaker enzyme should be used with caution because theoretically it can be shown that the overall rate of a system of several consecutive reversible reactions depends on the rate constants of each step. This objection is valid where the reactions are purely chemical and where no control is exerted over the activity and/or quantity of the catalysts. It should be recognized, however, that in a biochemical system the activity and quantity of the enzymes can be controlled by the genes (25); therefore, it is possible that the overall rate is controlled by one pacemaker enzyme.

The essence of the above discussion is that, if the pacemaker concept is correct, then the velocity of substrate assimilation via an enzymatic pathway would be given by the Michaelis-Menten equation of the pacemaker enzyme.

Kinetics of Cell Division and Growth

As a result of substrate assimilation, the mass of total protoplasm will increase in the culture and derivation of a rate law for growth of total biomass in the culture is of primary interest. This can be based upon the concept of growth yield discussed in Chapter II and the fact that microbes multiply through the process of binary fission. In a growing culture of initial cell concentration, $N_1$, at time, $\theta_1$, the cell concentration, $N_2$, at a later time, $\theta_2$, is given by

$$N_2 = N_1 2^n \tag{31}$$

where $n$ = number of cell divisions or generations occurring in the time interval, $(\theta_2 - \theta_1)$.

If $r$ is the number of cell divisions per unit time,

$$n = r(\theta_2 - \theta_1) \tag{32}$$

From Equations 31 and 32

$$N_2 = N_1 2^{r(\theta_2 - \theta_1)} \tag{33}$$

subtracting $N_1$ from Equation 33

$$\Delta N = N_1[2^{r\Delta\theta}-1] \tag{34}$$

where $\Delta N = N_2 - N_1$

In the limit of infinitesimal concentration and time differences, the differential equation for growth is obtained, or

$$\frac{dN}{d\theta} = (r \ln 2)N \tag{35}$$

Assuming $\rho^c$ to be the average content of mass per cell, and also that this factor does not change during the considered time interval of the growth phase, the following differential equation for growth in terms of mass density is obtained

$$\frac{dX^o_\theta}{d\theta} = (r \ln 2)X^o_\theta \tag{36}$$

where $X^o_\theta$ = biomass concentration (mass/unit volume of cell suspension) at any time $\theta$

Let $$r \ln 2 = k \tag{37}$$

Then from Equations 36 and 37

$$\frac{dX^o_\theta}{d\theta} = kX^o_\theta \tag{38}$$

where $k$ = the specific growth rate ($\text{time}^{-1}$)

Equation 38 is identical with Equation 2 in Chapter II. Since r is the "mean" cell division rate, k becomes the "mean" specific growth rate of all of the cells. The mean generation time $\bar{\tau}$ is defined as

$$\bar{\tau} = \frac{1}{r} \qquad (39)$$

so that from Equations 37 and 39

$$\bar{\tau} = \frac{\ln 2}{k} = 0.693/k \qquad (40)$$

As pointed out in Chapter II, the generation time, $\tau$, is given by a frequency distribution, $f(\tau)$, and the mean generation time is given by

$$\bar{\tau} = \int_0^{\alpha} \tau f(\tau) d\tau \qquad (41)$$

Painter and Marr (37) have shown that, for Gaussian distribution of $f(\tau)$ with a standard deviation, $\sigma$, Equation 41 can be solved to yield

$$\bar{\tau} = \frac{\ln 2}{k} + k\frac{\sigma^2}{2} \qquad (42)$$

It appears, therefore, that Equation 40, frequently used to calculate mean generation time, is not strictly correct as also indicated by Powell (35).

From consideration of conservation of mass and growth yield

$$dX_\theta^o = Y[dX_\theta^s]_g \qquad (43)$$

where $Y$ = true growth yield coefficient

$[dX_\theta^s]_g$ = substrate consumption for growth

From Equations 38 and 43 it follows that

$$k = \frac{1}{X_\theta^o} Y \frac{[dX_\theta^s]_g}{d\theta} \qquad (44)$$

If

$$fX_\theta^s = X_\theta^{sE} \qquad (45)$$

then

$$\frac{[dX_\theta^s]_g}{d\theta} = \frac{1}{f} \frac{dX_\theta^{sE}}{d\theta} \qquad (46)$$

where $X_\theta^s$ = concentration of extracellular substrate

$X_\theta^{sE}$ = concentration of the substrate catabolite which is the substrate of the pacemaker enzyme E

$f$ = a constant < 1

Substituting Equations 46 and 28 into Equation 44

$$k = \frac{1}{X_\theta^o} Y \frac{V}{f} \qquad (47)$$

For a given culture density (i.e., for fixed quantity of enzymes), the specific growth rate is maximum when V, the velocity of the pacemaker reaction, is maximum, or from Equation 47

$$k^m = \frac{YV^m}{X_\theta^o f} \qquad (48)$$

where $k^m$ = the maximum specific growth rate constant.

Rearranging Equation 48

$$\frac{Y}{X^{o}_{\theta}} = \frac{k^{m}f}{V^{m}} \tag{48A}$$

Substituting Equation 48A and Equation 29 into Equation 47

$$k = \frac{k^{m}X^{sE}}{K_{m}+X^{sE}_{\theta}} \tag{49}$$

but, $X^{sE}_{o} = fX^{s}_{o}$

so that,

$$k = \frac{k^{m}X^{s}_{\theta}}{K_{m}/f+X^{s}_{\theta}} \tag{50}$$

$$\text{If } K_{m}/f = K \tag{51}$$

then Equation 50 reduces to

$$k = \frac{k^{m}X^{s}_{\theta}}{K+X^{s}_{\theta}} \tag{52}$$

From Equation 51 it is obvious that the saturation constant, K, is higher than the Michaelis constant, $K_{m}$.

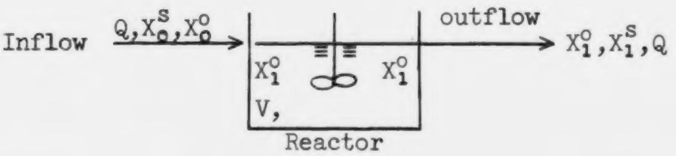
Recognizing that the exponential growth rate in certain cases is determined by a master (meaning pacemaker) reaction, Monod (7,38) adopted Equation 52 as an empirical law of exponential growth, because it bore similarity to the Michaelis-Menten equation. It is evident from the above that Monod's equation can be derived if one is allowed to assume that the concept of the master reaction is valid. Apart from the theoretical justification presented earlier in this Chapter, Equation 52 has been found applicable for the analyses of numerous experimental data, thereby supporting the validity of assuming a rate controlling enzymatic step in its derivation. After an extensive review of the information accumulated on the subject of enzyme response mechanisms and activity, Pardee (143) concluded that the concept of adjustment of all enzyme reactions to keep pace with the slowest reaction is tenable in view of the capacity of the cell to regulate the syntheses and activities of all enzymes. It can therefore be concluded that the Monod model is firmly based on the universal law of enzyme kinetics. However, since the master reaction approach is valid under conditions of steady state, the equation is most applicable for describing growth in the exponential phase. Furthermore, the equation is based on the premise that a constant fraction of the substrate, defined by the yield coefficient, is converted to biomass. Death rate is neglected. Available evidence indicates that these assumptions are valid for the exponential phase for all practical purposes.

## Differential Equations for Bacterial and Substrate Concentrations in Completely Mixed Continuous Flow Reactor

### Steady State Organism and Substrate Concentration

Mathematical models can be developed for concentrations of substrates and organisms in the reactor by applying the principles of mass balance to the biological system in the completely mixed reactor. A problem with this approach is that failure to account for all the inputs and outputs of the process would lead to models unable to realistically describe the parameters that the models represent. The mass balance of Monod (227), Novick and Szilard (73,228), and Herbert, et al. (240) did not include substrate consumption due to energy of maintenance, decrease in cell mass due to death and lysis, additional growth due to consumption of lysed products, decrease in cell mass due to predation, etc. As a result, the equations developed by these authors were unable to describe experimental data in many cases. Subsequently, Pirt (68), Spicer (75), and Saidel (81) amongst others have revised the mass balance equations to include some of the above factors. Additional revisions may be forthcoming as further insight is gained into other aspects of microbial metabolism.

With reference to the following didactic sketch, the equation for organism balance in a completely mixed continuous reactor may be written as



Didactic Sketch

$$\begin{array}{c}\text{Change in}\\ \text{reactor}\end{array} = \begin{array}{c}\text{Input of organisms}\\ \text{with inflow}\end{array} + \text{Growth} - \begin{array}{c}\text{Organism lost}\\ \text{in effluent}\\ \text{by dilution}\end{array} \qquad (53)$$

$$- \text{Death} + \text{Regrowth} - \text{Predation}$$

$$V\frac{dX_1^o}{d\theta} = X_0^o Q + k(X_1^s)X_1^o V - X_1^o Q - \delta(X_1^s, X_1^t, X_1^o)X_1^o V + \Phi\cdot\delta(X_1^s, X_1^t, X_1^o)X_1^o V \quad (54)$$

$$- (k^p(X_1^o)X_1^p)\frac{1}{Y^p}$$

where $X_1^o$ = concentration of active organisms in the reactor and effluent

$X_o^o$ = concentration of organisms in the influent

$X_1^s$ = concentration substrate in the reactor and effluent

$V$ = volume of reactor

$Q$ = flow-through rate

$k(X_1^s)$ = the specific growth rate constant which is a function $X_1^s$ and of a mathematical form to be defined later

$\delta(X_1^s, X_1^t, X_1^o)$ = death rate coefficient assumed to be a function of substrate concentration, $X_1^s$ (81) concentration of toxic metabolites, $X_1^t$ (13,81), crowding due to organism concentration (42,53), and number of cells (81,42)

$\Phi$ = coefficient of regrowth (42,53), i.e., ratio of cell divisions per number of cells dying

$k^p(X_1^o)$ = specific growth rate of predator organisms as a function of concentration of prey organisms, $X_1^o$

$X_1^p$ = concentration of predator organisms in the reactor and effluent

$Y^p$ = growth yield coefficient of the predator, i.e., mass of predator produced per unit mass of prey consumed

It has been stated by Postgate and Hunter (42) that the number of dead organisms may be substantial at low dilution rates when population density is high. The observation appears to be compatible with the fact that, with slower rates of flushing of toxic products and more crowding at lower dilution rates, the death rate may be of considerable magnitude at very high detention times. However, at higher dilution rates, death rates should be low for the accumulation of toxic metabolites is minimal. Herbert, et al. (240), Monod (227), Powell (58), and others chose to neglect death rate, while Saidel (81), Button and Garver (261), Hetling and Washington (110), Spicer (75), and Martin and Washington (241) preferred to include a term in the organism balance to account for the decrease in active biomass due to death. Postgate and Hunter (42) have indicated that the coefficient of regrowth is very small and since lysis, providing substrate for regrowth, occurs only under the extreme stresses of starvation not usually encountered in continuous reactors, the term for regrowth may be neglected. Population decrease due to predation may also be neglected as no significant predator population can be maintained except at low dilution rates. Due to their characteristic lower growth rates (61,313), predators get washed out at higher dilution rates. Since the nature of the function $\delta(X_1^s, X_1^t, X_1^o)$ is not known, the death rate coefficient is assumed to be a constant and independent of specific growth rates. Assuming that the influent contains no active organisms, Equation 54 can be reduced to

$$V\frac{dX_1^o}{d\theta} = k(X_1^s)X_1^oV - X_1^oQ - k'X_1^oV \qquad (55)$$

where $k' = \delta(X_1^s, X_1^t, X_1^o)$

At steady state, $dX_1^o/d\theta = 0$, and recognizing that $V/Q$ = the nominal detention time, $\theta_r$, Equation 55 becomes

$$k(X_1^s) = \frac{1}{\theta_r} + k' \qquad (56)$$

The mass balance for substrate concentration in a completely mixed continuous flow reactor is given by the equation

$$\begin{array}{c}\text{Change in}\\\text{reactor}\end{array} = \text{Input} - \text{Output} - \begin{array}{c}\text{Consumption}\\\text{by organisms}\\\text{for growth}\end{array} - \begin{array}{c}\text{Consumption by}\\\text{organisms for}\\\text{energy of}\\\text{maintenance}\end{array} + \begin{array}{c}\text{Amount}\\\text{contributed}\\\text{by lysis}\end{array} \qquad (57)$$

or,

$$V\frac{dX_1^s}{d\theta} = X_o^s Q - X_1^s Q - \frac{k(X_1^s)X_1^o V}{Y} - mX_1^o V + q\delta(X_1^s, X_1^t, X_1^o)X_1^o V \qquad (58)$$

where $X_o^s$ = substrate concentration in the influent

$m$ = *maintenance coefficient* in terms of substrate consumed per unit mass of organism per unit time

$Y$ = true yield coefficient = $dX^o/(dX^s)_g$

$q$ = substrate released per unit of biomass lysed (mg substrate/mg of biomass lysed)

In the above mass balance, a fraction of substrate assimilated, $(dX^s)_g$, is considered to account for the growth of active cells. The other fraction of substrate removal supplying the energy requirement is not recovered as cells but is destroyed by biological oxidation to yiel energy. The contribution to reactor substrate by lysis can be neglected under the operating conditions of this investigation. Therefore, at steady state, Equation 58 reduces to

$$\frac{X_o^s - X_1^s}{\theta_r} = X_1^o\left[\frac{k(X_1^s)}{Y} + m\right] \qquad (59)$$

Substituting for $k(X_1^s)$ from Equation 56

$$X_1^o = \frac{(X_o^s - X_1^s)Y}{1+\theta_r k' + mY\theta_r} \tag{60}$$

Rearranging Equation 60

$$\frac{X_o^s - X_1^s}{X_1^o} = \frac{1}{Y} + \theta_r \left[\frac{k' + mY}{Y}\right] \tag{60A}$$

It may be noted that $(X_o^s - X_1^s)/X_1^o$ is the total substrate consumption per unit biomass in the reactor. It is thus evident from Equation 60A that substrate consumption per unit biomass is a linear function of detention time, since Y, m, and $k'$ have been shown to be constants (68,75,81). Since $X_1^o/(X_o^s - X_1^s)$ is the apparent or observed yield coefficient, $Y^{o\,a}$, in terms of active solids, so that

$$\frac{1}{Y^{o\,a}} = \frac{1}{Y} + \theta_r \left[\frac{k' + mY}{Y}\right] \tag{61}$$

It is often of interest to know the mass of substrate utilized for growth of unit mass of cells synthesized. A constant, $U_s$, to be referred to as the substrate utilization factor may be defined to express mass of substrate utilized for growth of unit biomass.

Thus

$$U_s = \frac{1}{Y} \tag{62}$$

and Equation 61 becomes

$$\frac{1}{Y^{o}_{a}} = U_s + \theta_r\left[\frac{k' + mY}{Y}\right] \tag{63}$$

It should be noted that Equations 60, 60A, 61, and 63 can be derived without the form of $k(X_1^s)$ being specified. Equation 60 is independent of the Monod growth equation, and its failure to describe experimental data (see Curves d, c, and g of Figure 8) cannot be ascribed to any inadequacy of the Monod equation as has been alleged by some authors.

If $k(X_1^s)$ follows the Monod growth equation, then from Equations 52 and 56

$$\frac{k^m X_1^s}{K + X_1^s} = \frac{1}{\theta_r} + k' \tag{64}$$

Rearranging,

$$X_1^s = \frac{K(1 + k'\theta_r)}{\theta_r k^m - (1 + k'\theta_r)} \tag{64A}$$

and

$$\frac{\theta_r}{1 + k'\theta_r} = \frac{1}{X_1^s}\left[\frac{K}{k^m}\right] + \frac{1}{k^m} \tag{64B}$$

Equation 64B indicates that $\theta_r/(1+k'\theta_r)$ is a linear function of the reciprocal of substrate concentration.

## Auto-destruction or Decay Constant

Equations 60A and 61 are not the common forms used by many authors (91,95,96,98,99) in the sanitary engineering field who do not use the latest concept of energy of maintenance but rather the older and perhaps fictitious concept of negative growth proposed by Herbert (59). Since the negative growth is mathematically equivalent to the growth that would take place (but actually does not) if the substrate consumed for energy of maintenance is used for growth, then the following relationship can be written:

$$(Y)\,\frac{[dX_1^s]_m}{d\theta} = k^e X_1^o \tag{65}$$

In Equation 65 $[dX_1^s]_m$ is the energy substrate consumed for supplying the energy of maintenance so that the left hand side of this equation is equal to the fictitious rate of biomass production from $[dX_1^s]_m$, and is to be corrected by a hypothetical rate of negative growth given by $k^e X_1^o$. From the definition of the maintenance coefficient

$$\frac{[dX_1^s]_m}{d\theta} = mX_1^o \tag{66}$$

From Equation 65 and Equation 66

$$mX_1^o Y = k^e X_1^o \tag{67}$$

and

$$mY = k^e \tag{68}$$

Substituting Equation 68 into Equation 60A yields

$$\frac{X_1^s - X_1^s}{X_1^o} = \frac{1}{Y} + \theta_r\left[\frac{k' + k^e}{Y}\right] \tag{69}$$

Let

$$k' + k^e = k^d \tag{70}$$

It should be noted that the users of $k^d$ assume it to be a constant and independent of the specific growth rates.

Substituting Equation 70 into Equation 69

$$\frac{X_o^s - X_1^s}{X_1^o} = \frac{1}{Y} + \theta_r \frac{k^d}{Y} \qquad (71)$$

From Equations 68 and 70

$$k^d = k' + mY \qquad (72)$$

Equation 71 is the form used by many authors (91,95,96,98,99) who variously refer to $k^d$ as the specific "destruction" rate or "decay" rate to include decrease in biomass concentration due to endogenous respiration. However, it should be recognized that "endogenous" respiration in the presence of substrate does not involve degradation of cellular reserves (such as β-hydroxybutyrate, lipids, glycogen, etc.)(40,41,42, 53), and as such it is not proper to include this factor in the organism balance. In the presence of extracellular substrate, "endogenous respiration"--or more appropriately the requirement of basal metabolism or the energy of maintenance (40,71)--involves the exergonic oxidation of the substrate, and it should be accounted for in the substrate balance as shown in Equations 57 and 58.

The values of $k^d$ reported in the literature (see Table 1) are typically high, amounting to more than 50 percent of the maximum specific growth rate. If $k^d$ is interpreted to mean negative growth rate or decay or destruction of biomass, it is not understood how one can justify such high rates of organism decay or destruction in the presence of the substrate and under the controlled environmental conditions of continuous cultures. However, the values of $k^d$ appear to have some significance if this parameter is taken to be the sum of the specific death rate, $k'$, and the term, mY, as defined by Equation 72. Moreover, as $\underline{k}^d$ itself has hardly any significance in the absence of starvation conditions, it is more meaningful to use equations which incorporate the more realistic parameters of the specific death rate and the maintenance coefficient.

Table 1. Comparison of Auto-destruction Rates and Maximum Specific Growth Rates

| Author | $k^d$ (day$^{-1}$) | $k^m$ (day$^{-1}$) | Type of Culture |
|---|---|---|---|
| Gates, *et* *al*.(95) | 0.07 | 0.14 | Heterogeneous, anaerobic-aerobic |
| Gates, *et* *al*.(95) | 0.14 | 0.23 | Heterogeneous, anaerobic-aerobic |
| Andrews and Pearson(96) | 0.87 | 1.33 | Heterogeneous, anaerobic |
| Schroepfer, *et* *al*. (315) | 0.17 | 0.24 | Heterogeneous, anaerobic |
| Schroepfer, *et* *al*. (315) | 0.13 | 0.06 | Heterogeneous, anaerobic |

## Approximate Forms of the Steady State Equations

From inspections of Equations 60A and 64B, it may be realized that these equations involve too many unknown constants ($k'$, Y, m, $k^m$, and K) to be determined from the experimental data on steady state organisms and substrate concentrations.

Most authors have chosen to neglect k′ and considered the following simplified forms of Equations 56, 60, 60A, 61, 64A, and 64B:

$$k(X_1^s) = \frac{1}{\theta_r} \tag{73}$$

$$k(X_1^s) = D \tag{74}$$

$$X_1^o = \frac{(X_o^s - X_1^s)Y}{1+mY\theta_r} \tag{75}$$

$$X_1^o = \frac{(X_o^s - X_1^s)YD}{D+mY} \tag{76}$$

$$\frac{(X_o^s - X_1^s)}{X_1^o} = \frac{1}{Y} + m\theta_r \tag{77}$$

$$\frac{X_o^s - X_1^s}{X_1^o} = \frac{1}{Y} + \frac{m}{D} \tag{78}$$

$$\frac{1}{Y^{oa}} = \frac{1}{Y} + m\theta_r \tag{79}$$

$$\frac{1}{Y^{oa}} = \frac{1}{Y} + \frac{m}{D} \tag{80}$$

$$X_1^s = \frac{K}{\theta_r k^m - 1} \tag{81}$$

$$X_1^s = \frac{KD}{k^m - D} \tag{82}$$

$$\left[\frac{K}{k^m}\right]\frac{1}{X_1^s} + \frac{1}{k^m} = \theta_r \tag{83}$$

$$\left[\frac{K}{k^m}\right]\frac{1}{X_1^s} + \frac{1}{k^m} = 1/D \qquad (84)$$

Because detention time, $\theta_r$, is the reciprocal of the hydraulic dilution rate, D, Equations 74, 76, 78, 80, 82, and 84 are alternate forms of Equations 73, 75, 77, 79, 81, and 83, respectively. From Equations 73 and 79

$$\frac{1}{Y^{o}a} = \frac{1}{Y} + \frac{m}{k} \qquad (85)$$

Equation 85 was Pirt's (68) modified form of the equation derived earlier by Herbert, et al. (240). The above simplified equations allow the determination of constants, Y, m, $k^m$, and K, from the slopes and intercepts of the two lines best fitting the plots of $(X_o^s - X_1^s)/X_1^o$ and $\theta_r$, as functions of $\theta_r$ and $1/X_1^s$, respectively.

Critical Detention Time and Critical Dilution Rate. The upper limit of the hydraulic dilution rate and the lower limit of the detention time, respectively called the critical dilution rate, $D_c$, and the critical detention time, $\theta_r^c$, may be derived from Equations 81 and 82 when $X_o^s$ equals $X_1^s$. At critical detention times, the hydraulic dilution rate equals the growth rate, and there will be "washout" or no growth in the reactor at the expense of the incoming substrate. The approximate forms for $D_c$ and $\theta_r^c$ are as follows:

$$\theta_r^c = \frac{K+X_o^s}{X_o^s k^m} \qquad (86)$$

$$D_c = \frac{k^m X_o^s}{X_o^s + K} \qquad (87)$$

Substrate Conversion Rate. Economical process operation demands that the detention time be selected to yield the maximum substrate conversion rate, $C_r^m$. The conversion rate, $C_r$, is given by Equation 88 below:

$$C_r = D(X_o^s - X_1^s) \tag{88}$$

Eliminating $X_1^s$ in Equation 88 by Equation 82, differentiating the resultant expression for $C_r$ with respect to the dilution rate, D, and equating the differential to zero, the following expression for $\theta_r^{mc}$ is obtained

$$\theta_r^{mc} = \frac{(X_o^s+K)^{\frac{1}{2}}}{k^m[(X_o^s+K)^{\frac{1}{2}} - K^{\frac{1}{2}}]} \tag{89}$$

where $\theta_r^{mc}$ = detention time at which maximum conversion rate is realized. From Equations 89 and 81 it can be shown that

$$[X_1^s]_{\text{at max conversion rate}} = [K(X_o^s+K)]^{\frac{1}{2}} - K \tag{90}$$

It should be appreciated, however, that as $X_o^s$ increases, $\theta_r^{mc}$ approaches $\theta_r^c$ resulting in larger values of $X_1^s$. At a high influent concentration of the substrate, operation for maximum conversion rate will result in higher substrate concentration in the effluent. In sanitary engineering processes, the effluent quality is frequently of primary importance, and in such cases, efficiency of conversion has to be sacrificed for the sake of better effluent quality.

## On the Techniques Used for Determinations of the Growth Constants without Neglecting Death Rate

It has been shown by some investigators that the death rate is a function of substrate concentration, concentration of toxic metabolites, and crowding as a result of cell density (13,42,81). The factors influencing death become considerably important in reactors fed with high growth yielding substrates such as glucose and when operated at high detention times; thus, neglecting death seems to be unjustified, unless proved otherwise, in such cases. Schichiji and Uematsu (316) have reported that the fraction of dead cells in continuous cultures reaches appreciable levels. Button and Garver (261) concluded that, at lower growth rates, dead and metabolically inactive cells were present. However, there remains the problem of determining specific death rate and the other constants ($Y$, $m$, $k^m$, and $K$) from the experimentally measurable quantities and Equations 60A and 64B. The difficulty can be obviated by determining $\underline{k'}$ from measured concentrations of active bacterial solids and those of total solids, which may include dead and inactive cells as well as cell debris. In this investigation, the dehydrogenase activities were taken as a measure of the active solids concentration, $X_1^o$. An equation could then be derived for determining the specific death rate, $k'$, by performing a mass balance on total solids as follows:

$$\begin{array}{l}\text{Change in}\\ \text{completely mixed}\\ \text{continuous flow}\\ \text{reactor}\end{array} = \begin{array}{l}\text{Input}\\ \text{with}\\ \text{influent}\end{array} - \begin{array}{l}\text{Loss}\\ \text{with}\\ \text{outflow}\end{array} + \begin{array}{l}\text{Addition to}\\ \text{total solids}\\ \text{due to}\\ \text{growth}\end{array} \qquad (91)$$

If there are no solids in the influent, then

$$V\frac{dX_i^{o\,t}}{d\theta} = 0 - X_i^{o\,t}Q + kX_i^oV \qquad (91A)$$

where $X_i^{o\,t}$ = total dry solids concentration in the reactor

At steady state,

$$\frac{X_i^{o\,t}}{X_i^o} = k\theta_r \qquad (92)$$

Substituting for $k\theta_r$ from Equation 56

$$\frac{X_i^{o\,t}}{X_i^o} = 1+k'\theta_r \qquad (93)$$

$$X_i^{o\,t} = X_i^o(1+k'\theta_r) \qquad (93A)$$

From Equation 93 it is evident that the specific death rate constant, $k'$, is equal to the slope of a plot of $X_i^{o\,t}/X_i^o$ as a function of $\theta_r$. Comparison of the slope of the line best fitting the data on $X_i^{o\,t}/X_i^o$ and $\theta_r$ with the theoretical slope of unity would provide information regarding the reliability of this technique of measurement of $k'$. Equation 93A can be used to compute sludge production at selected detention times from the concentration of active solids as determined by dehydrogenase activities.

Having thus determined $k'$, the true yield coefficient, Y, and the maintenance coefficient, m, can be computed from the intercept and slope of the best line of fit of data plotted in accordance with the linear function of Equation 60A. The maximum specific growth rate constant, $k^m$, and the saturation constant, K, can be determined similarly from a plot of the experimental data in accordance with Equation 64B.

## Fractional Use of Assimilated Substrate for Growth and Maintenance

Mathematical expressions can be derived for estimating the fraction of assimilated substrate diverted for growth, and the fraction utilized for the energy of maintenance. The following equation, obtained by rearranging Equation 60A, describes how the assimilated substrate is partitioned for satisfying the different important biological functions.

$$1 = \frac{X_1^o}{Y}\left[\frac{1}{X_o^S - X_1^S}\right] + \frac{X_1^o}{Y} k'\theta_r\left[\frac{1}{X_o^S - X_1^S}\right] + m\theta_r X_1^o\left[\frac{1}{X_o^S - X_1^S}\right] \qquad (94)$$

In Equation 94

$\frac{X_1^o}{Y}$ = quantity of substrate consumed for growth per liter of culture

$X_1^o \kappa' \theta_r$ = mass concentration of cells which die at detention time, $\theta_r$

$\frac{X_1^o k' \theta_r}{Y}$ = mass of substrate consumed per liter of culture in producing cells which eventually die at detention time, $\theta_r$, and

$(X_o^S - X_1^S)$ = total substrate consumption by a liter of culture.

Thus,

$$\frac{X_1^o}{Y}\left[\frac{1}{X_o^S - X_1^S}\right] = \text{fraction of total substrate removal used for growth} \qquad (95)$$

$\frac{X_1^o}{Y}k'\theta_r\left[\frac{1}{X_o^s-X_1^s}\right]$ = fraction of total substrate removal that accounts for the growth of cells which eventually die at detention time, $\theta_r$ (96)

and

$m\theta_r X_1^o\left[\frac{1}{X_o^s-X_1^s}\right]$ = fraction of total substrate removal used for supplying the energy of maintenance at detention time, $\theta_r$ (97)

Considering that $X_1^o/(X_o^s-X_1^s) = Y^{oa}$, the following alternate forms of Equations 95, 96, and 97 can also be written:

$Y^{oa}/Y$ = fraction of total substrate removal used for growth (98)

$k'\theta_r\, Y^{oa}/Y$ = fraction of total substrate removal used for growth of cells which die at detention time, $\theta_r$ (99)

$m\theta_r Y^{oa}$ = fraction of total substrate removal used for supplying the energy of maintenance at detention time, $\theta_r$ (100)

## Population Dynamics in Continuous Flow Reactors

The phenomenon of selection of dominants in Chemostat type reactors has been discussed at length in Chapter II. The purpose of this section is to present a few relationships which can aid in ascertaining if several species can simultaneously survive at a chosen dilution rate. The effect of microbial interactions (such as commensalism, mutalism, amensalism, parasitism, predation, symbiosis, synergism, etc.) is not considered in the analysis presented herein.

If there are j species of microorganisms in the reactor operated at a dilution rate, D, then the growth rate of each species is given by Equation 56. Thus

$$k'+D = k_j(X_1^s) \tag{101}$$

where $j = 1,2,3,\ldots.n.$

If the specific growth rate is given by the Monod equation, then for n species

$$k'+D = k_1^m \frac{X_1^s}{K_1+X_1^s} = k_2^m \frac{X_1^s}{K_2+X_1^s} = \cdots\cdot = k_j^m \frac{X_1^s}{K_j+X_1^s} = \cdots\cdot = k_n^m \frac{X_1^s}{K_n+X_1^s} \tag{102}$$

where $X_1^s$ = substrate concentration in the reactor operated at dilution rate, D

$k_1^m, k_2^m, \cdots\cdot k_j^m$ = maximum specific growth rates of species Nos. 1, 2, ⋯j

$K_1, K_2, \cdots\cdot K_j$ = saturation constants of species Nos. 1, 2, ⋯j

All species can coexist at a given dilution rate of D if either Equation 103, 104, or 105 below is satisfied:

$$k_1^m = k_2^m = \cdots\cdot = k_j^m = \cdots\cdot = k_n^m \tag{103}$$

$$K_1 = K_2 = \cdots\cdot = K_j = \cdots\cdot = K_n \tag{104}$$

$$\frac{k_1^m}{K_1+X_1^s} = \frac{k_2^m}{K_2+X_1^s} = \cdots\cdot\frac{k_j^m}{K_j+X_1^s} = \cdots\cdot\frac{k_n^m}{K_n+X_1^s} \tag{105}$$

If it is assumed that Species No. 1 can survive at a dilution rate, D, then coexistence of Species No. 1 and Species j is possible when

$$k_1^m = k_j^m \tag{106}$$

and

$$K_1 = K_j \tag{107}$$

i.e., when the two species considered have identical growth kinetic properties. Species No. 1 and Species j may also grow together, albeit Equations 106 and 107 do not hold, if

$$\frac{k_1^m}{K_1+X_1^s} = \frac{k_j^m}{K_j+X_1^s} \tag{108}$$

Rearranging Equation 108

$$\frac{k_1K_j - k_jK_1}{k_j^m - k_1^m} \equiv X_1^s \tag{108A}$$

Again, for Species j

$$\frac{k_j^m X_1^s}{K_j+X_1^s} = D+k' \tag{109}$$

Since Species No. 1 is the reference species which is assumed to establish itself at dilution rate, D, $X_1^s$ is given by an expression of the form of Equation 64A, or

$$X_1^s = \frac{K_1(D+k')}{k_1^m-(D+k')} \qquad (110)$$

Substituting Equation 110 into Equation 109 yields

$$\frac{k_j^m K_1 \ (D+k')}{K_j k_1^m + (D+k')(K_1-K_j)} = (D+k') \qquad (111)$$

Rearranging Equation 111

$$\frac{k_j^m K_1 - k_1^m K_j}{K_1-K_j} = D+k' \qquad (112)$$

Thus, Species j may attain a steady state population with Species No. 1 if either of the conditions of Equations 108A or 112 holds. Finally, Equations 103, 104, 108A, and 112 define conditions under which two species can coexist in a completely mixed continuous flow reactor.

Having established the conditions for coexistence, it is a simpler matter to derive the conditions under which a test Species j cannot survive at a given reactor detention time or a steady state substrate concentration. The ability of the test species for survival is tested by comparing its growth kinetic parameters with those of a reference species known to be able to proliferate at the given dilution rate or reactor substrate concentration. If the growth kinetic parameters of the test Species j are such that

$$D+k' > \frac{k_j^m X_1}{K_j+X_1^s} \qquad (113)$$

i.e.,

$$D > k_j - k' \qquad (114)$$

then

$$\frac{k_j^m X_1^s}{K_j + X_1^s} - (D+k') = \text{negative quantity} \qquad (115)$$

If a mass balance for Species j is performed in the manner of Equation 55,

then

$$\frac{d\overset{o}{X}_1{}^j}{d\theta} = k(X_1^s)\overset{o}{X}_1{}^j - \overset{o}{X}_1{}^j(D+k') \qquad (116)$$

If the specific growth rate, k, is defined by Equation 52,

then

$$\frac{d\overset{o}{X}_1{}^j}{d\theta} = \overset{o}{X}_1{}^j\left[\frac{k_j^m X_1^s}{K + X_1^s} - (D+k')\right] \qquad (117)$$

From Equations 115 and 117 it is evident that

$$\frac{d\overset{o}{X}{}^j}{d\theta} = \text{negative} \qquad (118)$$

Thus, when $D > (k_j - k')$, i.e., when the dilution rate is greater than the net specific growth rate of Species j at a substrate concentration, $X_1^s$, dictated by Species No. 1, Species j will be progressively washed out; no steady state can be reached until Species j is completely eliminated. The time of washout depends on the concentration, $\overset{o}{X}_1{}^j$, present initially and the magnitude of $D-(k_j-k')$, which may be termed as the selection pressure.

Since $X_1^s$ is determined by Species No. 1, which is assumed to survive at the dilution rate, D, it follows from Equation 64 that

$$D+k' = \frac{k_1^m X_1^s}{K_1 + X_1^s} \tag{119}$$

Substitution of Equation 119 into Equation 113 yields

$$\frac{k_1^m X_1^s}{K_1 + X_1^s} > \frac{k_j^m X_1^s}{K_j + X_1^s} \tag{120}$$

In addition, substitution of Equation 110 into Equation 113 results in

$$(D+k') > \frac{k_j^m K_1 (D+k')}{K_j k_1^m + (D+k')(K_1 - K_j)} \tag{121}$$

The following relationships can now be defined on rearranging the Inequalities 120 and 121

$$\frac{k_j^m K_1 - k_1^m K_j}{K_1 - K_j} - k' < D \qquad (\text{when } K_1 > K_j) \tag{122}$$

$$\frac{k_1^m K_j - k_j^m K_1}{K_j - K_1} - k' > D \qquad (\text{when } K_j > K_1) \tag{123}$$

$$\frac{k_j^m K_1 - k_1^m K_j}{k_1^m - k_j^m} < X_1^s \qquad (\text{when } k_1^m > k_j^m) \tag{124}$$

$$\frac{k_1^m K_j - k_j^m K_1}{k_j^m - k_1^m} > X_1^s \qquad (\text{when } k_j^m > k_1^m) \tag{125}$$

Should any of the conditions of Inequalities 122 through 125 be applicable, then Species j would be progressively washed out and a steady state population of this species cannot be attained. It is apparent from relationships 122 and 123 that, as D is increased, the magnitude of the expression on the left hand side of the relationship would fall below D for an increasing number of species with the result that, at higher dilution rates, the total population would be composed of fewer species than would prevail at low dilution rates. Conversely, as the dilution rate is decreased, conditions given by Inequalities 122 and 123 would be applicable for an increasing number of species, thereby resulting in increased heterogeneity of population. Therefore, theoretically the heterogeneity of the reactor population will increase with decrease of dilution rate or increase of detention time. If the steady state substrate concentration in the reactor in the presence of a species of defined growth kinetic properties is known, then Inequalities 124 and 125 can be used to ascertain if another Species j can survive under these conditions.

# CHAPTER V

# DEVELOPMENT AND DESIGN OF THE EXPERIMENTAL APPARATUS

## Completely-mixed Continuous Flow Reactor System for Heterogeneous Bacterial Cultures

### General

Despite the fact that numerous designs and assemblies have been described in the literature for the continuous cultivation of microorganisms in the laboratory and on a commercial scale, there is hardly any particular design which would be satisfactory for all of the diverse objectives which could be considered. The design of the system details would vary according to the purpose for which the system is to be used. For example, a good apparatus for production of vitamins may not be suitable for the study of mutation or ideal for kinetic studies or even desirable for mass production of cells.

Compared to applications in pure culture studies, not many continuous reactor assemblies have been described for studies of heterogeneous cultures. A basic difference, which is rather an advantage, of the latter type of studies is that the need for sterilizing equipment and keeping it sterile during operation for maintaining the purity of the culture is eliminated. However, a noteworthy disadvantage is the unavoidable slime formation on the surfaces of the reactor and its appurtenances. As will be discussed in a later section, failure to keep slime formation under control may make the difference between meaningless and meaningful data.

The difficulties of attaining a stable steady state condition in heterogeneous culture have already been mentioned in Chapter II. The fluctuations in substrate and organism concentrations during a steady state run would be amplified by a large degree if slime growth, sloughing, and flow oscillations associated with the use of pumps for delivery of nutrients were allowed in the operation. These factors should be eliminated as far as possible.

It was decided to design an inexpensive completely mixed continuous culture system, capable of being assembled from standard items of laboratory equipment and hardware, which would eliminate the use of pumps but would render reliable long-term service. Other desirable features would include the provision for alteration of culture volume from run to run (or even during a run, if desired) and inclusion of a standby system for continued operation during any power shutdown.

### Design Considerations

The design of the system was based upon the following general functional requirements:

a) provision for adequate agitation of the culture to obtain complete mixing and homogeneity;

b) provision for adequate oxygen transfer rate;

c) provision for maintaining the pH at a desired level;

d) means of controlling and maintaining the temperature at a selected value;

e) means of delivering the nutrients at a constant but easily variable rate;

f) means of controlling the concentrations of nutrients and keeping concentrations constant during a run;

g) means of maintaining constant culture volume but with provisions for variations;

h) provisions for batch preparation and storage of nutrient solutions for delivery to the reactor at constant measured rates.

For purposes of design and operation, the continuous culture system was considered to be comprised of the following subsystems:

a) Reactor assembly
b) Mixing device
c) Temperature control
d) Air supply
    (i) for mixing and oxygen transfer during normal operation
    (ii) for mixing and oxygen transfer in operation during power shutdown (operation during emergencies)
    (iii) for pressurizing feed reservoirs and effecting substrate and nutrient delivery against positive lifts
e) Feed reservoirs and appurtenances
f) Continuous flow system
g) Oxygen supply to reactor
h) Foam control
i) pH control

## Design of the Reactor Assembly

Reactors of various shapes, sizes, and materials of construction and ranging from a simple laboratory flask (261,317) to the Porton-type vessel of specialized engineering construction have been reported (134, 135,318). The materials chosen for construction of the reactor and appurtenances should be relatively inexpensive, durable, resistant to corrosion under a wide range of pH and oxidative conditions, non-toxic, rigid and not easily breakable, transparent, easily workable, immune to damage from moderate mechanical and thermal shocks, and should have a minimum affinity for slime growth compared to glass and stainless steel. Cast plastic sheets and tubes are inexpensive, transparent, of light weight, durable, inert towards chemicals usually encountered in laboratory cultures and involve a minimum of labor and skill for building an apparatus. This material can be easily glued, sawed, drilled, tapped, sanded, and machined. This synthetic plastic, however, has the disadvantage that it dissolves in organic solvents and is attacked by strong acids.

The reactor, waterbath, feed reservoirs, and air filters were made of cast thermoplastic acrylic resin sheets and tubes. Equipment and appurtenances which were to be immersed in the culture were of stainless steel, Teflon, rubber, or plastic as indicated on Figures 10 and 11.

Since slime growth was expected in the heterogeneous culture, the reactor was constructed with a minimum of edges and surface areas and a parallelopiped shape. The capacity of the reactor was designed to provide the desired number of samples of sufficient volume without significantly affecting the culture volume (and therefore the detention time) and the oxygen transfer rate. By using the method of Perret (319), the minimum allowable reactor volume was computed to be 4.5 liters (see Appendix I).

The reactor dimensions were chosen so that an operating volume

**LEGEND**

SS - 1/2" DIA STAINLESS STEEL SHAFT
C - 1/2" COLLAR
BA - BAFFLE
W - WING NUT FOR CLAMPING OVERFLOW SUPPORT TO REACTOR WALL.
TS - FRAMED SUPPORT FOR OVERFLOW CUP
OC - OVERFLOW CUP
Y - "Y"-SHAPED GLASS OVERFLOW TUBE
TC - TUBE CLEANER
RE - RE-ENTRANT ENTRANCE WITH ABRUPT CONTRACTION
TO - 1" I.D. TELESCOPIC OVERFLOW PIPE
O - "O"-RING
T - THERMOMETER
I - IMPELLER
WO - WEIR OVERFLOW
SSB - STAINLESS STEEL BOLT

NOTE: ALL DIMENSIONS ARE IN INCHES.
REACTOR, BAFFLES, OVERFLOW ASSEMBLY & IMPELLER WERE MADE OF PLEXIGLASS 3/8" OR 1/4" THICK.

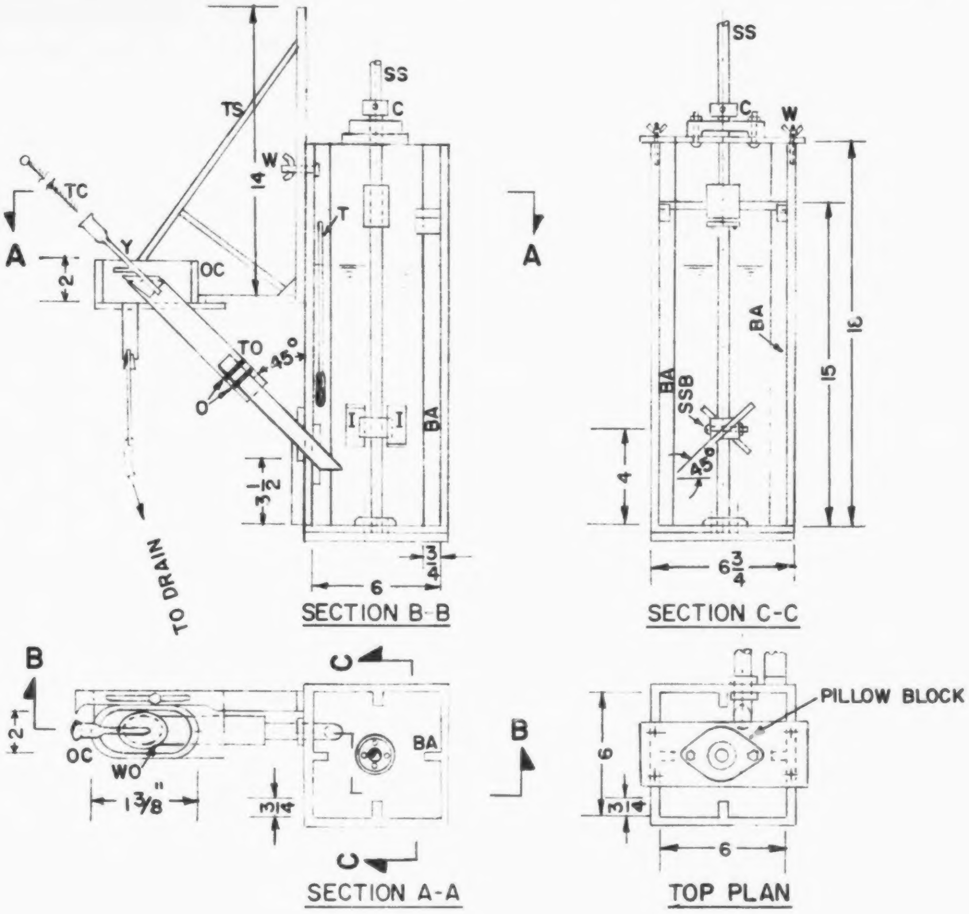
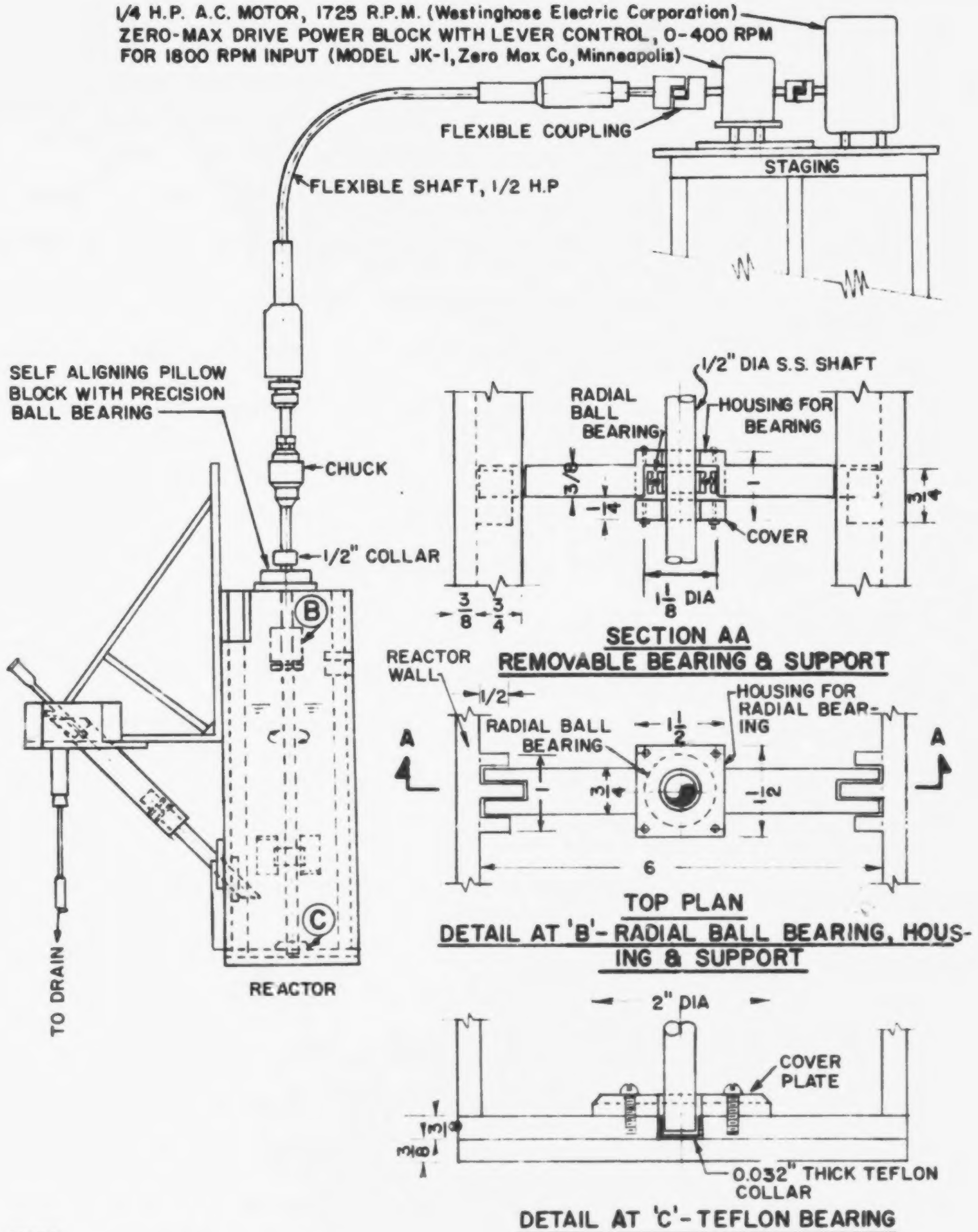

SS
C
TS
W
TC
Y
A
2
OC
14
T
TO
45°
O
I
I
BA
3 1/2
TO DRAIN
6
3/4
SECTION B-B
A
SS
C
W
BA
BA
SSB
45°
4
15
13
6 3/4
SECTION C-C
B
C
2
OC
WO
1 3/8"
3/4
BA
C
SECTION A-A
B
PILLOW BLOCK
6
3/4
6
TOP PLAN

FIGURE 10. DETAILS OF REACTOR ASSEMBLY & WEIR OVERFLOW


1/4 H.P. A.C. MOTOR, 1725 R.P.M. (Westinghose Electric Corporation)
ZERO-MAX DRIVE POWER BLOCK WITH LEVER CONTROL, 0-400 RPM FOR 1800 RPM INPUT (MODEL JK-1, Zero Max Co, Minneapolis)
FLEXIBLE COUPLING
STAGING
FLEXIBLE SHAFT, 1/2 H.P
SELF ALIGNING PILLOW BLOCK WITH PRECISION BALL BEARING
CHUCK
1/2" COLLAR
B
C
REACTOR
TO DRAIN
1/2" DIA S.S. SHAFT
RADIAL BALL BEARING
HOUSING FOR BEARING
3/8
1/4
3/4
COVER
1 1/8 DIA
3/8
3/4
SECTION AA
REMOVABLE BEARING & SUPPORT
REACTOR WALL
1/2
HOUSING FOR RADIAL BEARING
RADIAL BALL BEARING
1 1/2
A
A
3/4
1/2
6
TOP PLAN
DETAIL AT 'B'- RADIAL BALL BEARING, HOUSING & SUPPORT
2" DIA
COVER PLATE
3/8
3/8
0.032" THICK TEFLON COLLAR
DETAIL AT 'C'- TEFLON BEARING

NOTE.

1. ALL DIMENSIONS ARE IN INCHES
2. 3/8" & 1/4" PLEXIGLAS USED FOR CONSTRUCTION

FIGURE 11. DETALS OF THE MECHANICAL MIXING DEVICE

up to seven liters could be attained. To minimize slime growth, the number of projections and appurtenances projecting into the culture fluid was kept to a minimum. The reactor dimensions are also shown in Figure 10.

### Control of the Reactor Volume

Operation of the reactor at a constant detention time (or dilution rate) required that the culture volume be maintained constant during each run. The devices used by Perret (319), Herbert, et al. (320), DeHaan and Winkler (321), and others (318,261) suffer from various disadvantages such as the use of complex equipment and operating procedures, outflow in discrete discontinuous slugs, etc. Moreover, these devices do not allow any variation of the culture volume. The wier overflow system used in this research and illustrated in Figure 10 was of such construction that a steady outflow rate could be obtained, and the culture volume could be varied at will at any time. A telescopic tube entering the reactor just below the impeller at an angle of 45 degrees was sufficient to allow overflow without clogging. The oscillations of liquid or surge in the overflow tube were similar to the classical case of surge in a U-tube due to turbulent resistance. Although usually controlled by allowing the liquid to surge into a tank, the surge in the overflow tube was controlled by provision of a Y-shaped glass outlet of smaller diameter (see Figure 10). The rise of the effluent in the branch of the Y-tube and the accompanying losses aided in dissipating the momentum transferred to the overflowing fluid due to the vigorous agitation inside the reactor. The Y-tube also facilitated cleaning.

## Mixing Device

The purpose of stirring the culture fluid is to achieve complete mixing of the reactor contents and effect adequate oxygen transfer. Various stirring devices, such as diffused aeration (317,321), spinning the reactor (319), stirring with a magnetic mixer, and motor driven impellers (318,320) have been used and recommended. Since good results with mixing by mechanical stirring have been experienced (95,134,135, 318), this technique was chosen. The selected mixing device is shown in Figure 11. The stainless steel shaft was centered in the reactor through a self-aligning pillow block with a precision ball bearing and was suspended in the reactor by means of the collar to avoid grinding of the shaft against the reactor bottom. Inside the reactor, the central alignment was maintained by passing the shaft through a radial ball bearing above the fluid level and allowing it to rotate within a Teflon collar inserted at the floor of the reactor. The radial ball bearing was housed in a Plexiglas housing mounted on a removable support resting on the reactor wall. The impeller, mounted on the stainless steel shaft with a stainless steel bolt, consists of a solid block of Plexiglas with two plane rectangular vanes fixed on two opposite vertical sides of the block at an angle of 45° to the horizontal as shown in Figure 10. The reactor shaft was connected to a flexible drive shaft through a chuck. The stainless steel shaft, impeller, and the radial ball bearing enclosed within the housing and support could be removed whenever the reactor needed cleaning. The drive consisted of a one-fourth horsepower AC motor coupled to a zero-Max drive power block which allowed a range from very slow to high speed agitation.

Mixing and Oxygen Transfer in Emergencies. In the event of a power failure or shutdown, mixing of the culture and oxygen transfer were accomplished by vigorous bubbling of compressed air supplied through the standby system shown in Figure 12. As will be explained in the following section, the supply of compressed air also formed a part of the system for air supply needed to pressurize the feed reservoirs. The objective of the standby operation was to maintain continuity of operation at the prevailing dilution rate in the event of power failures.

### Temperature Control

Various temperature control systems were available commercially for automatic control and the selection was based on the temperature requirements of the culture mass, the thermal resistance of the reactor material, the temperature of the immediate atmosphere and its nature of fluctuation, the tolerable control cycle* and thermal lag,** and the availability and cost.

Since the laboratory was air conditioned and the heating and cooling rates were small, a thermocouple pyrometer with electric control was selected to provide satisfactory control of temperature. A constant temperature recirculating water bath equipped with a refrigerator compressor and a low lag heating coil which could control the temperature between -20°C to + 71°C was also chosen. As shown in Figure 13, the reactor temperature was controlled by circulating water at 20°C from the

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*Control cycle is the temperature excursion of the controlled mass about the control point.

**Thermal lag is that condition whereby a change in the rate of heat dissipated at one point in the controlled system is felt at other points after delays in time.

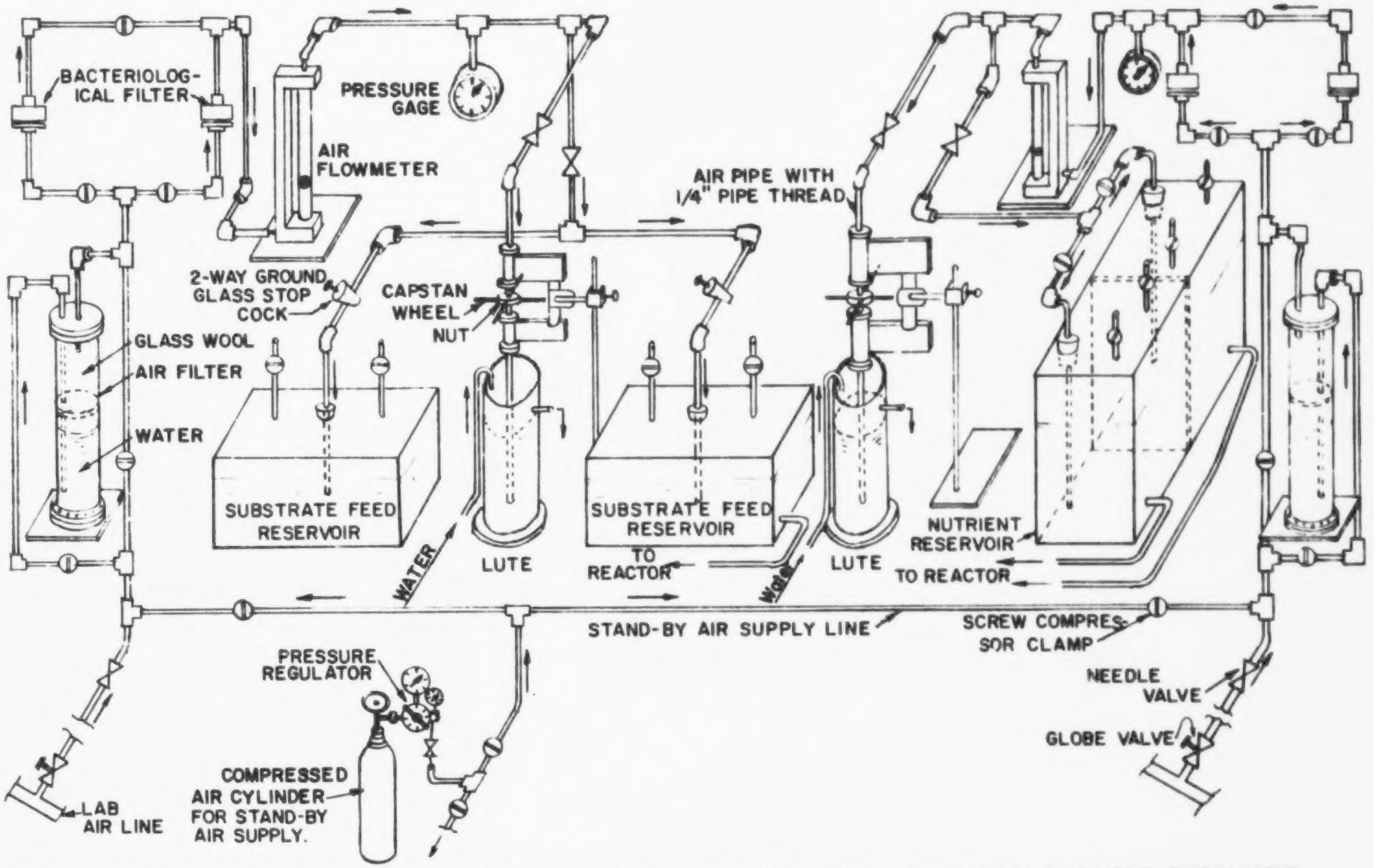

BACTERIOLOG-
ICAL FILTER
PRESSURE GAGE
AIR FLOWMETER
AIR PIPE WITH 1/4" PIPE THREAD
2-WAY GROUND GLASS STOP COCK
CAPSTAN WHEEL
NUT
GLASS WOOL
AIR FILTER
WATER
SUBSTRATE FEED RESERVOIR
WATER
LUTE
SUBSTRATE FEED RESERVOIR
TO REACTOR
Water
LUTE
NUTRIENT RESERVOIR
TO REACTOR
STAND-BY AIR SUPPLY LINE
SCREW COMPRES-SOR CLAMP
NEEDLE VALVE
GLOBE VALVE
PRESSURE REGULATOR
COMPRESSED AIR CYLINDER FOR STAND-BY AIR SUPPLY.
LAB AIR LINE

FIGURE 12. AIR SUPPLY SYSTEM FOR SUBSTRATE AND NUTRIENT DELIVERY AND FOR DIFFUSED AERATION DURING NORMAL AND EMERGENCY OPERATION.

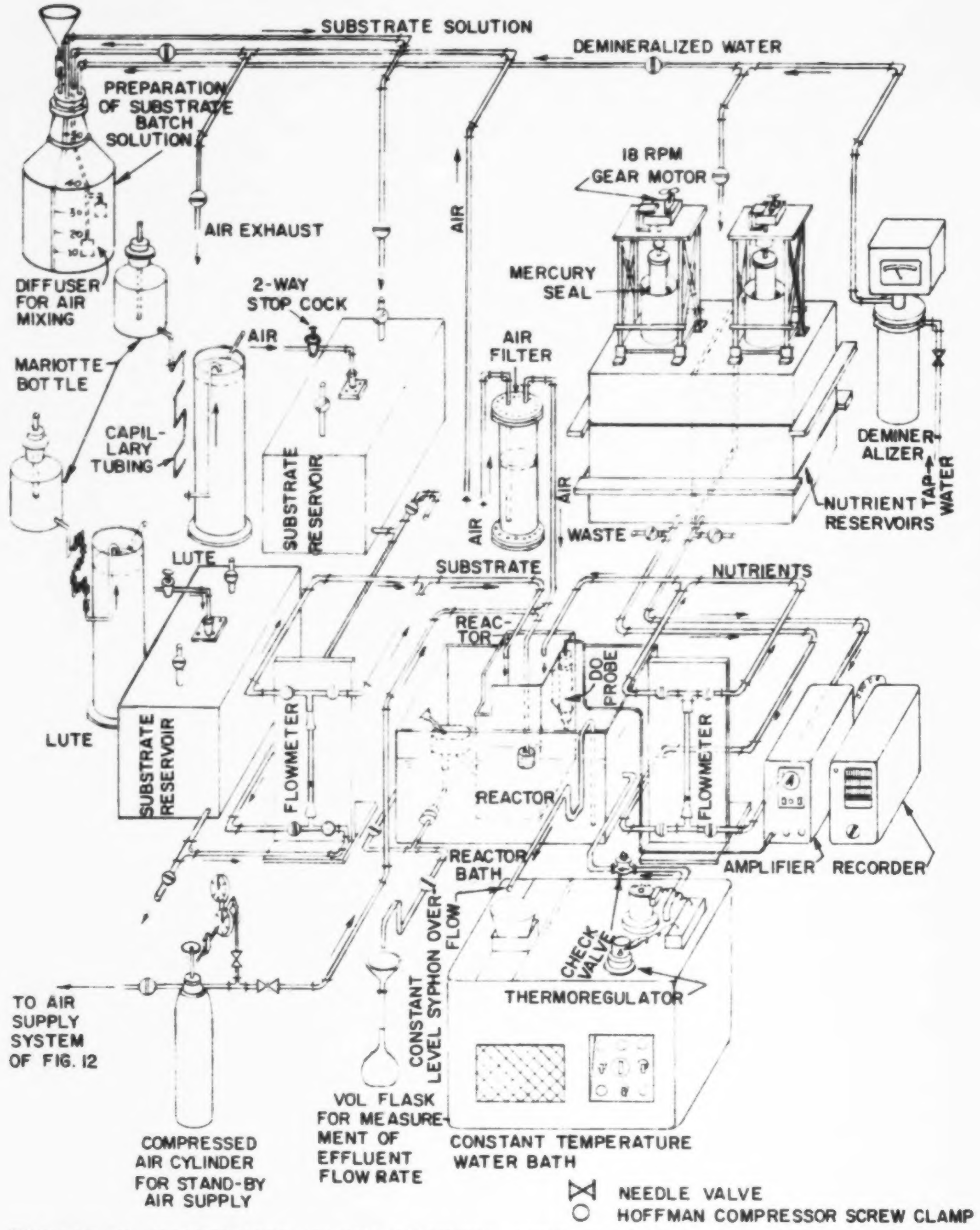

SUBSTRATE SOLUTION
DEMINERALIZED WATER
PREPARATION OF SUBSTRATE BATCH SOLUTION
AIR EXHAUST
AIR
18 RPM GEAR MOTOR
MERCURY SEAL
DIFFUSER FOR AIR MIXING
2-WAY STOP COCK
AIR
AIR FILTER
MARIOTTE BOTTLE
CAPILLARY TUBING
SUBSTRATE RESERVOIR
DEMINERALIZER
TAP WATER
AIR
AIR
NUTRIENT RESERVOIRS
WASTE
LUTE
SUBSTRATE
NUTRIENTS
REACTOR
DO PROBE
LUTE
SUBSTRATE RESERVOIR
FLOWMETER
REACTOR
FLOWMETER
AMPLIFIER
RECORDER
REACTOR BATH
CONSTANT LEVEL SYPHON OVER FLOW
CHECK VALVE
THERMOREGULATOR
TO AIR SUPPLY SYSTEM OF FIG. 12
VOL FLASK FOR MEASUREMENT OF EFFLUENT FLOW RATE
COMPRESSED AIR CYLINDER FOR STAND-BY AIR SUPPLY
CONSTANT TEMPERATURE WATER BATH
⋈ NEEDLE VALVE
○ HOFFMAN COMPRESSOR SCREW CLAMP

FIGURE 13. CONTINUOS FLOW SYSTEM, TEMPERATURE CONTROL, DIFFUSED AERATION, DO MONITORING SYSTEM AND THE EQUIPMENT FOR PREPARATION OF SUBSTRATE FEED SOLUTION.

constant temperature water bath to the reactor bath in which the water level was kept constant due to the provision of a constant level type of syphon outlet. Back-syphoning from the reactor bath to the constant temperature bath in case of any breakdown was prevented by provision of a check valve.

### Air Supply

Supply of air was needed mainly for two purposes: (1) to pressurize the nutrient feed reservoirs and (2) for oxygen supply to the culture when aeration by mechanical mixing was not adequate. The alternative system of mixing and oxygen supply by diffused aeration and pressurizing the feed reservoirs by compressed air has already been discussed in a previous section.

Figure 12 shows the system for supplying air to the nutrient reservoirs. The arrangements of lines, valves, and filters are identical for the supply of air to the nutrient (salts) and the substrate reservoirs. The pressure of the laboratory air supply was controlled by a globe valve at the laboratory outlet followed by fine control by a needle valve before the air entered into the system. The air was cleaned of oily materials, lubricants, etc. by first bubling through a water trap at the bottom of the air filter and then filtering out the particulate matters by passing it through the upper chamber of an air filter packed with glass wool.

The air cleaning and measuring system was mounted on a board. The purpose of the water trap in the air filter was to humidify the air and prevent evaporation loss in the reactor. The air supply to the reactor was humidified by fine bubbling through a fine diffuser inserted at the inlet end of the air pipe going into the lower chamber of the air filter (see Figure 14). However, it was necessary to clean the lower chamber of the air filter of greasy deposits from time to time and refill it with clean water to a depth of about one inch below the perforated disc separating the top glass wool chamber.

## Feed Reservoirs

Due to the heterogeneous nature of the microbial culture, it was not necessary to sterilize the nutrients. However, the salts and buffer solutions were separated from the substrate since otherwise there would have been growth of microorganisms inside of the nutrient reservoir and the nutrient supply lines. Such growth may have resulted in clogging of lines and an irregular decrease and variation of the concentration of the nutrient feed solutions to the reactor. Furthermore, under the assumptions made in the derivation of steady state equations of Chapter IV, there should not be any organisms present in the influent.

In order to facilitate continuous operation with a minimum of interruption to the medium flow, provisions were made for duplicate substrate and nutrient reservoirs. The capacity of each reservoir was based on the minimum expected dilution rate of one hour, a reactor volume of six liters, and a maximum frequency of two fillings of each reservoir in a 24 hour period. Each reservoir was provided with a nutrient outlet at the bottom, a drain, an inlet at the top for filling with prepared solution, an air tube, and an air exhaust at the top. The end of the glass air pipe was ground with carborundum stone to provide the square edges required for discrete bubble formation and their continuous and uniform rate of release. The flow of air into each

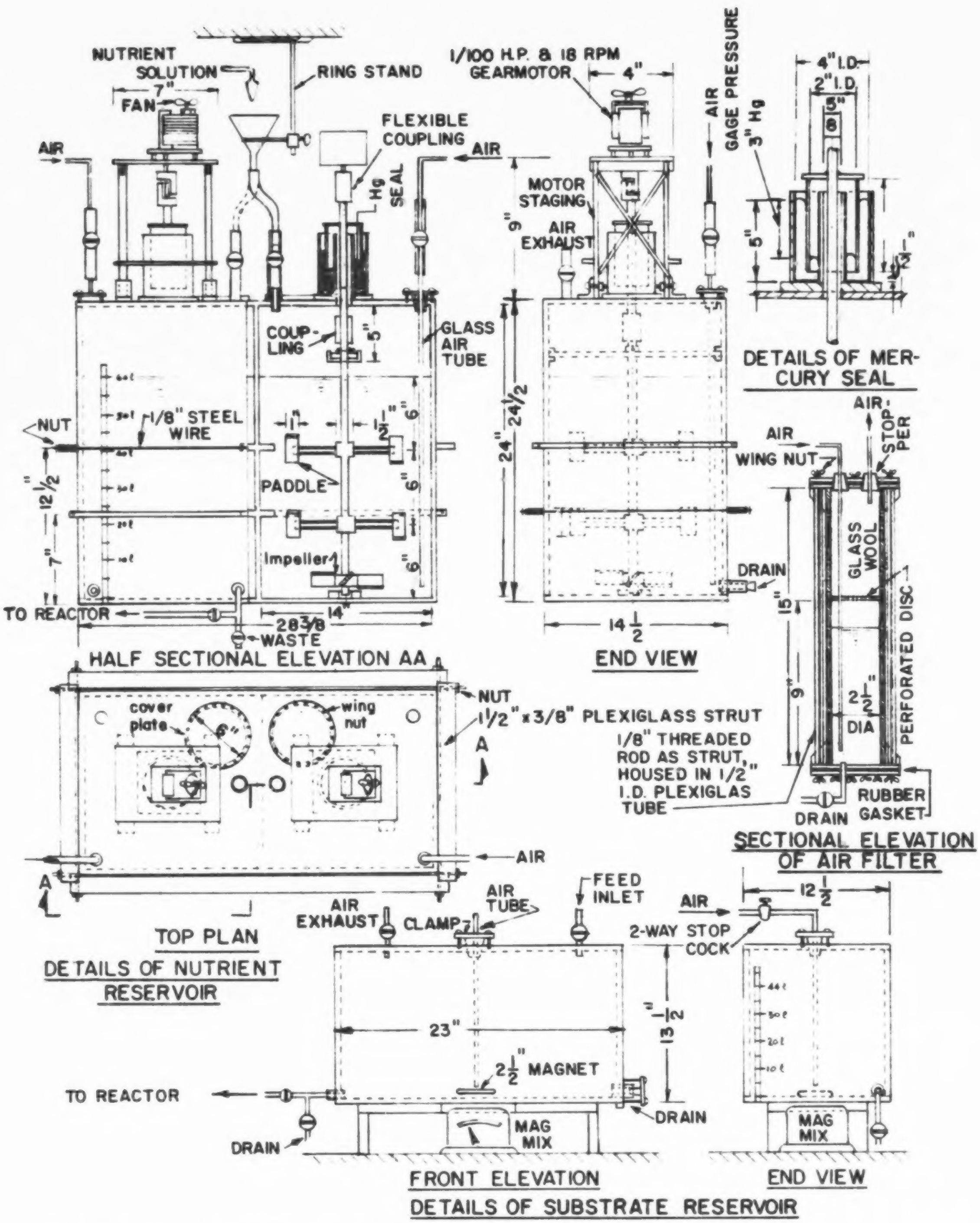

NUTRIENT SOLUTION
RING STAND
7"
FAN
AIR
FLEXIBLE COUPLING
AIR
Hg SEAL
COUP-LING
5"
GLASS AIR TUBE
NUT
1/8" STEEL WIRE
1"
1 1/2"
6"
PADDLE
6"
12 1/2"
7"
Impeller
6"
TO REACTOR
28 3/8
14"
WASTE
HALF SECTIONAL ELEVATION AA
1/100 H.P. & 18 RPM GEARMOTOR
4"
AIR GAGE PRESSURE
MOTOR STAGING
9"
AIR EXHAUST
24"
24 1/2
DRAIN
14 1/2
END VIEW
4" I.D.
2" I.D.
5"
8
3" Hg
5"
5"
2 1/2"
DETAILS OF MERCURY SEAL
AIR
AIR
STOPPER
WING NUT
GLASS WOOL
PERFORATED DISC
15"
9"
2 1/2" DIA
RUBBER GASKET
DRAIN
SECTIONAL ELEVATION OF AIR FILTER
NUT
cover plate
6"
wing nut
1 1/2" x 3/8" PLEXIGLASS STRUT
1/8" THREADED ROD AS STRUT, HOUSED IN 1/2" I.D. PLEXIGLAS TUBE
A
A
AIR
TOP PLAN
DETAILS OF NUTRIENT RESERVOIR
AIR EXHAUST
AIR TUBE
CLAMP
FEED INLET
AIR
2-WAY STOP COCK
12 1/2
23"
13 1/2"
2 1/2" MAGNET
TO REACTOR
DRAIN
DRAIN
MAG MIX
MAG MIX
FRONT ELEVATION
END VIEW
DETAILS OF SUBSTRATE RESERVOIR

FIGURE 14. DETAILS OF FEED RESERVOIRS AND AIR FILTERS

air tube was controlled with the aid of a two way ground glass stopcock or Hoffman screw clamp.

The contents of the substrate reservoirs were mixed with magnetic mixers. The salt solutions in the nutrient reservoirs were mixed with motor driven shaft and paddles. A mercury seal, designed for a maximum pressure differential of three inches of mercury, was provided around the point of entry of the shaft into the nutrient reservoir. The details of the construction of the nutrient reservoirs are shown in Figure 14.

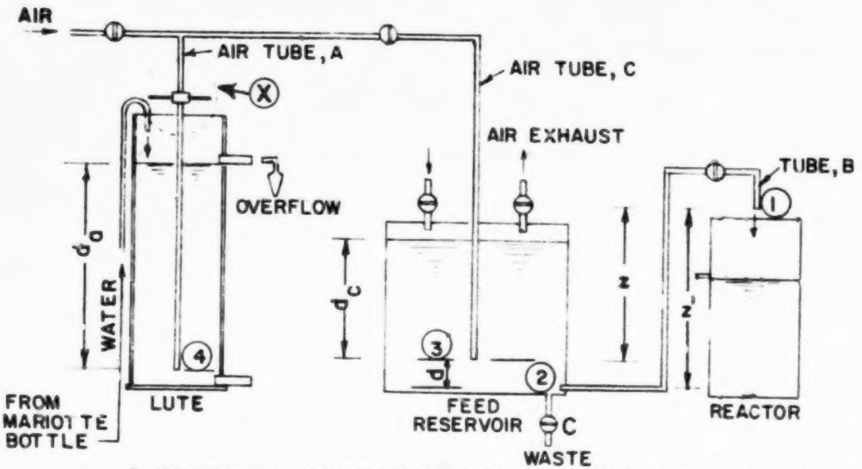
### Continuous Flow System

*General Considerations.* The continuous flow system was comprised of the feed reservoirs, devices for delivery of the nutrients from the reservoirs to the reactor at an accurately controlled but easily variable rate, instruments for measuring the flow rates, and the medium feed lines. The desirable features of the system included provisions for: (1) accurate control of the medium flow rate, (2) maintenance of a constant flow rate over a prolonged period of operation, and (3) varying the flow rate with ease.

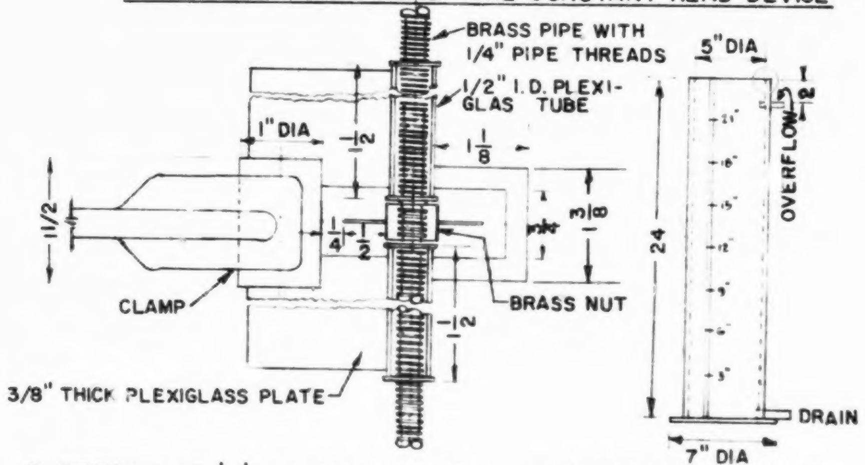
*Constant Head Device for Delivery of Nutrients and Substrates.* Delivery of the nutrients may be accomplished with the aid of pumps (usually a "Micropump" for small flows less than 1.5 liters per hour or "Peristaltic" pumps for higher flow rates), but there are some serious disadvantages associated with this procedure. Apart from electrical and mechanical breakdown, the main disadvantage of the use of pumps is long and short-term fluctuations and delivery by discontinuous spurts at lower flow rates. Clogging of tubes, inconsistent pumping rates at low flow rates, and rapid wear of tubings are the problems encountered by many investigators using Peristaltic pumps (320,134,135). Adjustment and maintenance of steady flow rates were difficult with Micropumps (320). It was felt that these factors would amplify the oscillations of steady state commonly encountered with heterogeneous cultures. The use of pumps was therefore deemed unsuitable for this research.

In order to eliminate the problems associated with nutrient supply by means of pumps, gravity flow systems have been used by some investigators (318,321,322). Gravity feed was considered preferable due to its simplicity, economy, minimum use of moving parts, and proven high degree of reliability during automatic operation over a prolonged period of time. For this research, a gravity system was designed to operate on a hydraulic principle which formed the basis of flow systems used by DeHaan and Winkler (321) and Elsworth, et al. (318).

The operational characteristics of the gravity flow system are shown in Figure 15A. A constant flow rate at Point 1 was attained by keeping the total hydraulic head at Point 2 constant during a steady state run. This was accomplished by sealing the nutrient reservoir from the atmosphere and inserting an air tube (Tube C) into the reservoir through an air tight joint. One branch of the air line (Tube A) extended into a column of water called the Lute which was provided with an overflow at a fixed level. The depth of immersion, $d_a$, of Tube A could be adjusted with a capstan wheel (see Figure 15A and 15B). The compressed air flowed into either Tube A or Tube C and could be controlled by means of the screw clamps shown in the figures. The system was so operated that the air Tube A in the Lute was constantly purged with a steady


AIR
AIR TUBE, A
X
AIR TUBE, C
AIR EXHAUST
TUBE, B
1
OVERFLOW
$d_a$
WATER
$d_c$
3
d
4
2
Z
Z'
LUTE
FROM MARIOTTE BOTTLE
FEED RESERVOIR
C
WASTE
REACTOR

A. SCHEMATIC DIAGRAM OF THE CONSTANT HEAD DEVICE


BRASS PIPE WITH 1/4" PIPE THREADS
1/2" I.D. PLEXIGLAS TUBE
1" DIA
$\frac{1}{2}$
$1\frac{1}{8}$
$1\frac{1}{2}$
$\frac{3}{8}$
$\frac{1}{4}$
$\frac{1}{2}$
CLAMP
BRASS NUT
$\frac{1}{2}$
3/8" THICK PLEXIGLASS PLATE
5" DIA
$\frac{1}{2}$
OVERFLOW
24
21"
18"
15"
12"
9"
6"
3"
DRAIN
7" DIA

B. DETAILS AT 'X', CAPSTAN WHEEL  C. DETAILS OF THE LUTE

FIGURE 15. CONSTANT HEAD DEVICE FOR DELIVERY OF SUBSTRATES AND NUTRIENTS.

stream of air bubbles at a flow rate of about 0.2 liters per minute.

The air tube in the Lute was threaded throughout its entire length with one-eighth inch pipe threads and could be lowered or raised by a fraction of a centimeter thereby changing the depth, $d_a$, by the same amount. With the aid of the device, the flow rate could be changed in very small increments or decrements of a few milliliters per hour. To prevent the subsidence of the Lute water level due to evaporation loss, water was allowed to drip into the Lute at a constant but very slow rate of approximately 10 ml/hr to overcompensate the evaporation loss. The excess flow was discharged through a constant level overflow. The system feeding the Lute operated in accordance with the Mariotte bottle* principle. Capillary tubes were interposed in the feed line to make the flow control very sensitive. The details of construction of the Lute are shown in Figure 15C.

Hydraulic analysis (see Appendix II) of the continuous flow system as schematically presented in Figure 15A yielded the following equation for the influent flow rate

$$Q_i = K(d_a - z)^{\frac{1}{2}} \tag{126}$$

where $Q_i$ = flow rate of feed solution into reactor

$d_a$ = depth of immersion of Tube A in the Lute (see Figure 15A) z = static head of outlet of Tube B above the open end (outlet) of Tube C (see Figure 15A)

---

*Mariotte bottle is an apparatus developed by the French scientist Edme Mariotte (1620-1684), co-discoverer of Boyle's law, to furnish a flow of water under a constant head equal to the height of the bottom of the adjustable vertical tube above the level of the outlet.

K = a constant as defined in Appendix II

From Equation 126 it was evident that, theoretically, a constant and steady flow rate could be obtained as long as the depth of the immersion of Tube A in the Lute and the elevation of the outlet of the feed Line B were kept constant.

Medium Feed Lines. Rubber, surgical blood transfusion type, and tygon tubings were tried as recommended by Herbert, et al. (320), but they were found unsuitable since they were clogged by air bubbles released from the substrate solution. The substrate solution in the reservoirs was supersaturated with air due to the prevalance of higher than atmospheric pressure and continuous bubbling. Upon emanating from the reservoir, the pressure gradually decreased to atmospheric pressure and tiny gas bubbles were released from the solution and adhered to the walls of the tubing. The tiny bubbles agglomerated into larger bubbles thereby blocking the flow and resulting in gradual reduction of the flow rate. An attempt to release air from the solution by heating the flowing solution at the point of exit from the reservoir and trapping the bubbles at the top of a vertical tube on the medium line proved unsuccessful. However, it was discovered that the accumulation of bubbles was markedly less in a section of the line built with glass tubing. Therefore, medium lines consisting of glass tubing virtually eliminated the problem of clogging of delivery lines with trapped bubbles. In the event of any sign of air bubble formation, addition of antifoam (Antifoam C emulsion for fermentation processes, Dow Corning Corporation, Midland, Michigan) in a concentration of two to four ppm ensured uninterrupted flow. Glass tubing also facilitated thorough cleaning by chromic acid solution. Alternate lines were provided for medium flow between the flow-measuring device and the outlet to eliminate interference to continuity of flow in the event that the line in use was to be cleaned. The layout of the nutrient feed lines is shown in Figure 13.

Measurement of Flow Rates. The constancy of the nutrient flow rate was continuously indicated by a laboratory flowmeter (Flowrator with one glass and one stainless steel ball float, Fisher and Porter Company, Hatboro, Pennsylvania) inserted in the medium flow line. The flow rate could be obtained from standard calibration curves after correction for deviations of fluid temperature, viscosity, and specific gravity from the standard conditions of calibration.

The resistance to flow through the capillary of the flowmeter changed due to deposition of very thin layers of slime growth frequently invisible to the naked eye. Coupled with the altered volume, surface area, specific gravity, and drag characteristics of the ball floats due to slime deposits, this resulted in erroneous flowmeter readings for known flow rates. It was therefore decided to measure the combined flow rates of substrate and nutrients by an arrangement such as the one shown in Figure 16. The flows of substrate and nutrients were received in a trough placed temporarily below the outlets, and the combined flow was diverted through the funnel and tubing for collection in a volumetric flask over a timed interval. The flowmeters mainly served as indicators of stability of flow rates. To provide relief from interruptions of

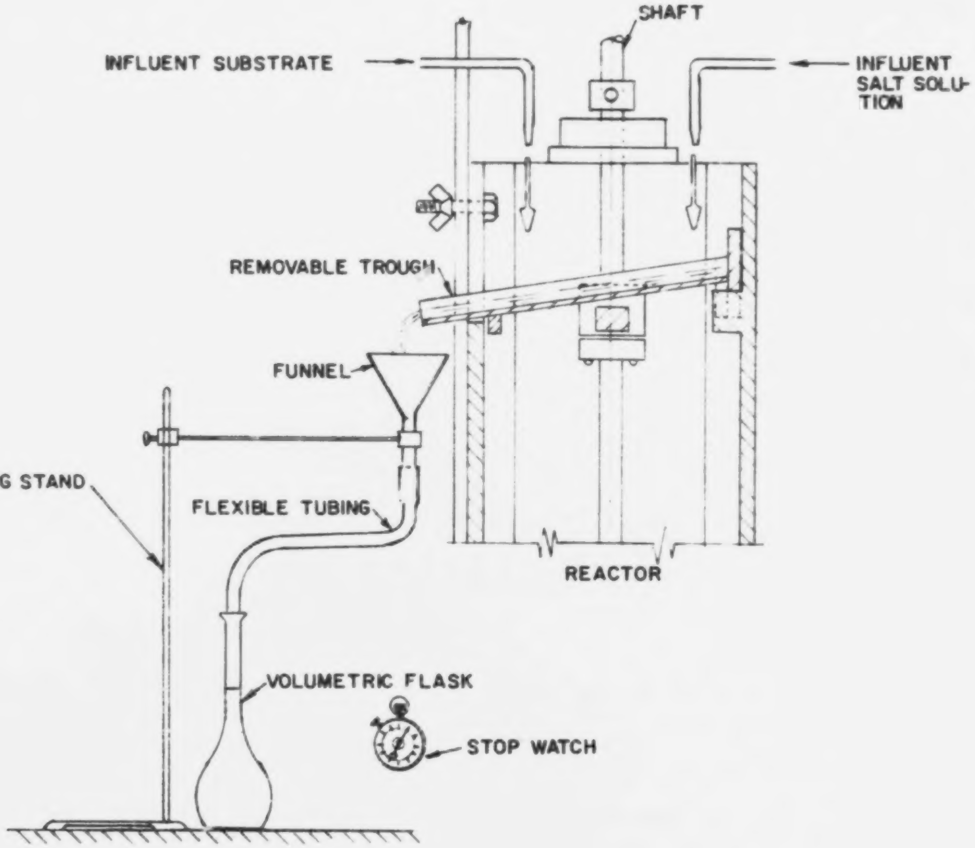

SHAFT
INFLUENT SUBSTRATE
INFLUENT SALT SOLUTION
REMOVABLE TROUGH
FUNNEL
RING STAND
FLEXIBLE TUBING
REACTOR
VOLUMETRIC FLASK
STOP WATCH

FIGURE 16. EQUIPMENT FOR DIRECT MEASUREMENT OF COMBINED FLOW RATES OF INFLUENT SUBSTRATE AND NUTRIENTS.

the influent flow caused by the above method of influent flow measurement, flow rates were also determined by collecting the effluent in a volumetric flask for a known period of time in a manner shown in Figures 13 and 17.

Oxygen Supply to the Reactor

Oxygen supply to the culture was effected by oxygen transfer by surface aeration due to the violent mixing conditions. At high dilution rates (i.e., at higher specific growth rates), the surface reaeration was not sufficient to satisfy the oxygen utilization rates of the cultures in cases where a high influent concentration of substrate with a resulting higher culture density was used. In such cases, surface transfer was supplemented with diffused aeration. When power was unavailable, oxygen was supplied entirely by diffused aeration. The systems for providing oxygen have been discussed in the preceding sections and are shown in Figures 12, 13, and 17.

As it was desirable to maintain DO levels upwards of one ppm (61) and since DO levels were also used to indicate steady state and transient conditions, it was decided to monitor and record DO levels continuously. A lead-silver galvanic cell oxygen analyzer developed by Mancy and Westgarth (323) was used as the oxygen sensor. The current output from the galvanic cell was amplified through an amplifier (Type 98 DC amplifier 1-10 μa, Rustrak Instrument Company, Inc., Manchester, New Hampshire) and recorded continuously with a recorder (miniaturized automatic chart recorder, one ma, Rustrak Instrument Company, Inc., Manchester, New Hampshire) at a chart speed of one inch per hour. The oxygen monitoring and recording system is shown in Figure 13.

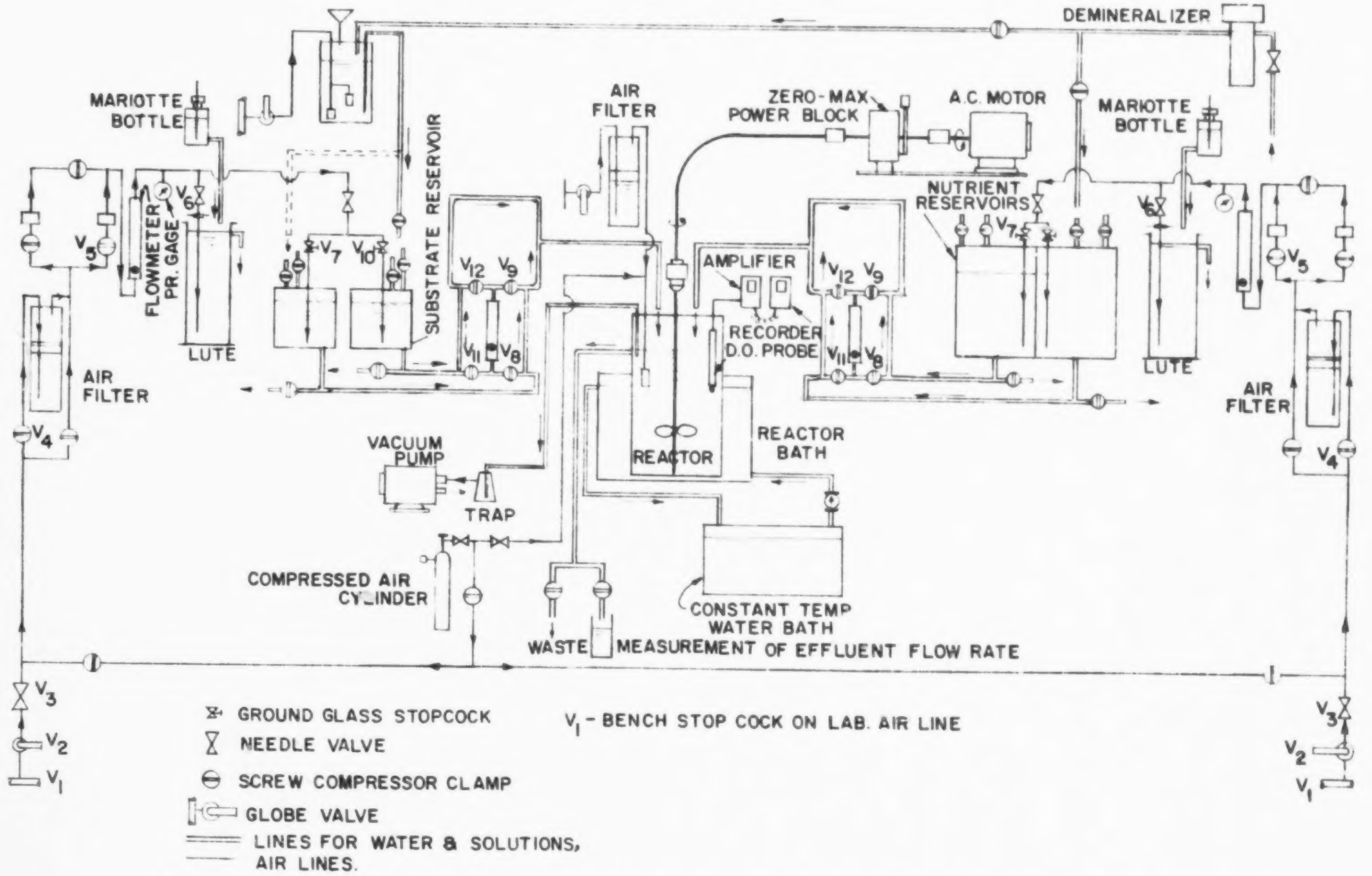

DEMINERALIZER
MARIOTTE BOTTLE
AIR FILTER
ZERO-MAX POWER BLOCK
A.C. MOTOR
MARIOTTE BOTTLE
NUTRIENT RESERVOIRS
FLOWMETER
PR. GAGE
LUTE
SUBSTRATE RESERVOIR
AMPLIFIER
RECORDER
D.O. PROBE
LUTE
AIR FILTER
AIR FILTER
VACUUM PUMP
TRAP
REACTOR
REACTOR BATH
COMPRESSED AIR CYLINDER
CONSTANT TEMP WATER BATH
WASTE
MEASUREMENT OF EFFLUENT FLOW RATE
$V_1$
$V_2$
$V_3$
$V_4$
$V_5$
$V_6$
$V_7$
$V_8$
$V_9$
$V_{10}$
$V_{11}$
$V_{12}$
GROUND GLASS STOPCOCK
NEEDLE VALVE
SCREW COMPRESSOR CLAMP
GLOBE VALVE
LINES FOR WATER & SOLUTIONS,
AIR LINES.
$V_1$ - BENCH STOP COCK ON LAB. AIR LINE

FIGURE 17. SCHEMATIC DIAGRAM OF THE CONTINUOUS CULTURE APPARATUS

## Foam Control

In microbiological and fermentation research, foaming has been traditionally controlled by the continuous or intermittent addition of antifoams to the culture through electrically controlled metering devices. A discussion of foam control with antifoams appears in a review by Elsworth, et al. (318).

Due to the absence of diffused aeration for oxygen transfer and mixing, serious foaming was not encountered during this research. The extent of foaming varied with detention times and culture density being highest at higher detention times. The accumulated foam could be satisfactorily removed by suction from time to time and the addition of antifoaming agents was not needed. The equipment for aspiration of foams is shown in Figure 18.

## pH Control

The maintenance of pH at the selected value or range can be accomplished either by buffering the medium (134,135) or by using automatic pH control as discussed by Callow and Pirt (324) and Lovelock and Nicholas (325). At the cell concentrations and metabolic rates encountered in this investigation, the pH value of the growth medium could be maintained at the desired range by suitably buffering the medium. The design of the buffer system is presented in Chapter VII.

## Operation of the Continuous Flow Reactor System

Figure 17 is a schematic diagram of the completely-mixed continuous flow reactor system. The following stepwise procedure for startup and maintenance of continuous flow was employed.

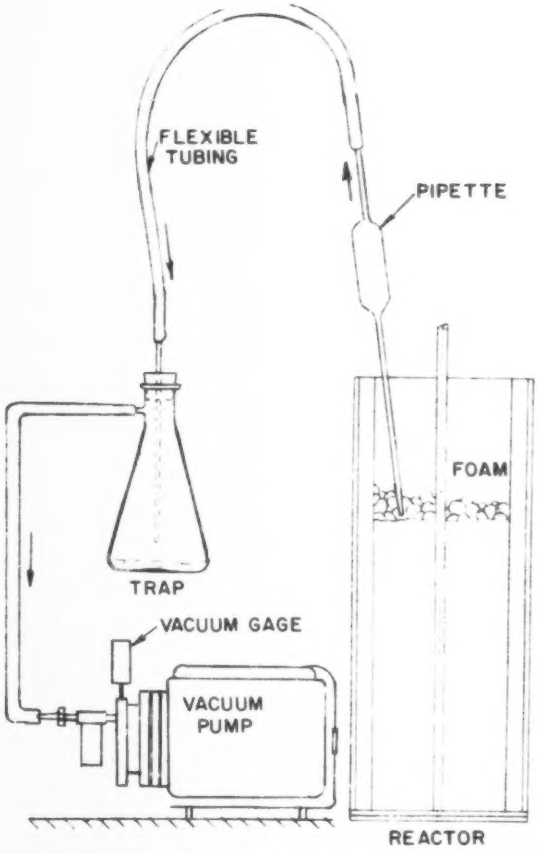

FLEXIBLE TUBING
PIPETTE
FOAM
TRAP
VACUUM GAGE
VACUUM PUMP
REACTOR

FIGURE 18. EQUIPMENT FOR REMOVAL OF FOAM FROM THE CONTINUOUS FLOW REACTOR.

1. Fill substrate and nutrient feed reservoirs. (Procedures for preparation of batches of substrate and nutrient solutions and filling the feed reservoirs are given in Chapter VII.)

2. Start drip-feeding the Lutes with tap water at about 10 ml/hr to compensate for evaporation loss and to maintain constant water level without unduly disturbing the water surface.

3. Clamp off the inlets and air exhaust outlets of substrate and nutrient feed reservoirs.

4. Allow the feed lines from the feed reservoirs to the reactor to be filled up with solutions, clamp to stop flow, adjust line outlets at suitable elevations above the reactor, and clamp to fix the selected position of the feed line outlets. Measure the heights of lift, z, for the substrate and nutrient delivery.

5. Adjust the volume of the reactor contents to the desired value by raising or lowering the overflow cup and then fixing it at the desired level by clamping the trussed support to the reactor wall.

6. Adjust the depths of the air tubes in the Lutes such that the depth in each Lute is about half of a centimeter larger than the corresponding lift, z.

7. Temperature control:

    a) adjust coarse control of the thermoregulator of the water bath at the desired value of temperature;

    b) begin recirculating water from the constant temperature water and back to the constant temperature water bath. Adjust the level of the syphon outlet from the reactor bath to obtain the desired water level in the reactor bath;

c) manipulate the fine control of the thermoregulator to arrive at the desired water temperature of the reactor contents.

8. Start mixing the reactor contents at a suitable level of agitation to provide complete mixing.

9. Suspend the DO probe into the culture and begin recording the DO. If the DO level is below 0.5 mg/$\ell$, provide supplemental oxygen transfer by diffused aeration.

10. Open valves $V_1$ through $V_6$ of the air supply system (see Figure 17) keeping the others closed and allow bubbles to escape from the air tube in the Lute. It should be emphasized that attainment of steady flow rate with the aid of the constant head device depends very much on proper operation of the Lute. It is important to keep the air flow rate at the minimum, but sufficient for the air pressure to just exceed the water pressure at the point of bubble formation. Bubbling should be characterized by discrete formation and escape of a series of bubbles in succession. Satisfactory control of air flow rate is achieved by throttling the needle valve. High air flow rates, causing a rapid stream of bubbles escaping at the end of the air tube in the Lute, produce pressure fluctuations which imperil the constancy of the flow of bubbles from the air tube in the feed reservoirs, ultimately manifested in oscillations of flow. This problem has been discussed at length by DeHaan and Winkler(321).

11. a) open Screw Clips $V_8$ and $V_9$ in the substrate feed line and Stopcock $V_7$ in the air tube in Substrate Reservoir 1 to allow flow to occur. Adjust depth of air tube in

Lute to achieve the desired flow rate. Note the flowmeter reading;

b) use same procedure as in 11(a) to obtain the desired rate of nutrient flow.

12. Maintenance during continuous operation:

a) keep Lute water depth constant. Check lines feeding water to Lute for clogging. Refill bottles supplying water to Lute;

b) check for clogging of substrate and nutrient feed lines and clean as necessary after switching to the alternate line;

c) check flowmeter reading, DO, pH, and temperature for constancy and make adjustments if necessary. Remove any foam by aspiration;

d) check flowmeter readings against direct flow measurements and clean flowmeter if readings do not check;

e) to supply substrate from the alternate Substrate Reservoir No. 2, close ground glass Stopcock $V_7$ (assuming Reservoir No. 1 was operating) and Screw Clips $V_8$ and $V_9$. Open ground glass Stopcock $V_{10}$ and Screw Clips $V_{11}$ and $V_{12}$ readjusting Lute water depth as necessary to maintain the existing flow rates. The details of the alternate feeding system were made very similar to each other so that the readjustment of depth of air tube in the Lute was accomplished by only a few turns of the capstan wheel. The interruption in the continuity of constant flow rate was not more than one minute. Diversion of nutrient supply from the alternate nutrient was done in a manner similar to the above procedure.

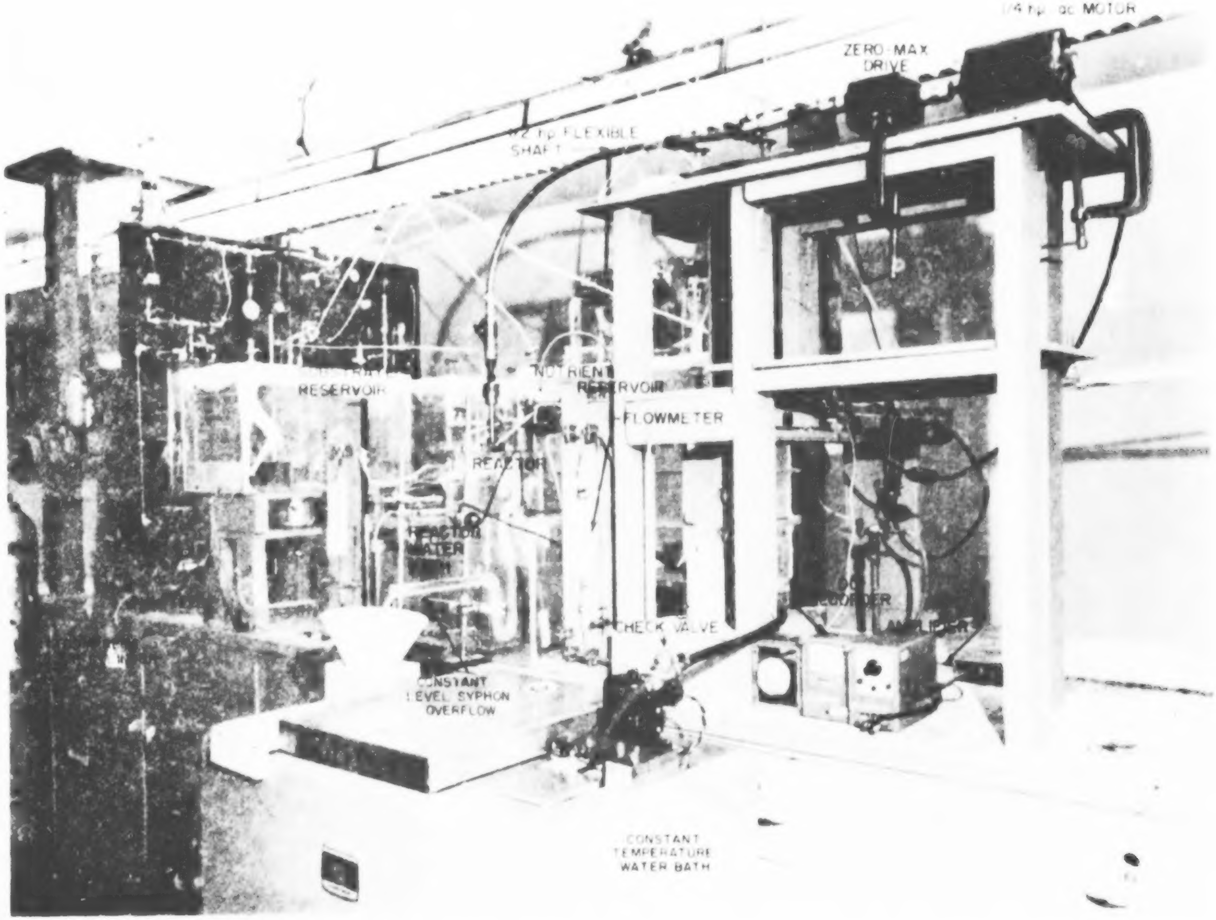

1/4 hp dc MOTOR
ZERO-MAX DRIVE
1/2 hp FLEXIBLE SHAFT
SUBSTRATE RESERVOIR
NUTRIENT RESERVOIR
FLOWMETER
REACTOR
REACTOR WATER BATH
DO RECORDER
AMPLIFIER
CHECK VALVE
CONSTANT LEVEL SYPHON OVERFLOW
CONSTANT TEMPERATURE WATER BATH

FIGURE 19. THE CONTINUOUS CULTURE APPARATUS

# CHAPTER VI

# DEVELOPMENT OF ANALYTICAL TECHNIQUES

## Introduction

While the detention times in the continuous flow reactor could be kept constant by following the procedure outlined in the last section of the preceding chapter, the course of the steady state runs was to be followed by measuring the substrate and the organism concentrations in the reactor. Since there are no satisfactory standard methods for determinations of active or viable organism concentrations, development of a satisfactory procedure for such measurements was necessary. It was also intended to develop a simplified and quick procedure for measurement of glucose and galactose concentrations ranging from one mg/ℓ or less to about 300 mg/ℓ. This chapter is devoted to the selection of suitable analytical techniques, discussion of the theoretical basis of the test techniques, and the development of detailed procedures for performing the selected tests.

## Development of an Analytical Procedure for the Measurement of Organism Concentration

### General Perspective

A tacit assumption involved in the mass balance approach to the derivation of the continuous culture theory was that the cell population in the reactor was biochemically *active*, i.e., they assimilated nutrients for: (1) synthesis of cellular materials, and/or (2) the energy of maintenance. Since the equations derived in Chapter IV theoretically describe steady state concentration of biochemically active biomass, it is desirable to adopt a technique of measurement which would differentiate between active and inactive biological solids in the reactor. Inactive solids would include any cell debris and other filterable solids of organic or inorganic character. The word active is preferred to the word viable[*] since the latter terminology is usually used in reference to microorganisms which do not multiply due to environmental factors not conducive to cell division. Such non-viable cells do, however, retain the ability to degrade substrates for deriving energy of maintenance during the stationary or declining growth phase as the work of Postgate and Hunter (42) and Harrison (53) would suggest. During the early lag phase there is virtually no cell division, but this is not to be construed as lack of any biochemical activity in the cell since the sizes of the cells increase (13) gradually in preparation for eventual fission into two daughter cells. Bearing this in mind, a few general remarks may be made about the techniques employed for estimation of active organism concentration.

## On Techniques for the Estimation of Active Biomass Concentration

The volatile solids content (327) which is very often used in sanitary engineering for estimating biomass concentration is not necessarily indicative of the active fraction of the total solids (328) since all volatile matter, organic or inorganic, would be included in the measurement irrespective of their biological or non-biological origins.

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[*] Tempest, et al. (326) have mentioned that living cells may be non-viable in the sense that they are capable of carrying out many of their metabolic processes but are not capable of reproducing themselves.

An apparently direct method, which is often used, involves the determination of the number of active cells per unit volume (cell concentration) by the standard plate count (327). Criticisms of this technique include the fact that the data obtained only give the number of cells capable of forming a colony on agar media under an environment perhaps very different from those prevailing in the culture. As pointed out by Monod, many organisms are extremely sensitive to sudden change in environment and often perish during and after transfer to the new environment. The accuracy of the data depends on the assumption of one cell forming one colony which of course is true only when the cells do not have any tendency to clump, and the cell suspensions used for plating are sufficiently dilute. To obtain mass density, the cell concentration has to be multiplied by the bacterial density which varies from one phase to another of the growth cycle (13,230,231). The procedure is tedious and involves long incubation periods. Quicker procedures with short incubation times have been developed by Powell (329) and Fung and Kraft (330). However, these latter methods would not work if a long lag is involved, and the results obtained would be of limited statistical accuracy.

Perhaps the most widely used method in quantitation of microbial growth is based on the determination of transmitted light through the cell suspension. Although the method is convenient and quick, a direct estimate of mass concentration is not possible unless a relationship between percent light transmittance and bacterial density is established. The light absorbing and light scattering properties of bacterial cultures change from one growth phase to another due to change in size, protoplasmic composition, and density of the cells. It is therefore doubtful that a constant linear relationship would exist between the optical density and the mass concentration at all specific growth rates. Also, meaningful measurement of optical density of flocculent suspensions is almost impossible due to the continuous change of physical properties of the suspension due to settling in the measuring cuvettes. For highly dispersed growth, it might be possible that optical density bears a linear relationship with bacterial density throughout the exponential phase. In any event, this measurement does not differentiate between the living or the dead or even inert colloids.

A more rational approach is to measure some physical, or biochemical, property of the culture. The selected parameter should definitely be indicative of the dynamic biochemical activities of the cell population and should be sensitive to changes in growth rates of the culture in such a manner that it bears a constant proportionality to mass concentration of cells at all growth rates. Respiration rate and concentrations of various cellular constituents have been thought to fulfill this requirement.

The respiration rate of a sample of biological culture under conditions of non-proliferation, often referred to as the endogenous respiration rate, has been used as a measure of active cells in the culture (328). The technique appears to have sound biochemical basis, but measurement of the parameter in the laboratory by the manometric or the polarographic technique (327) is extremely time consuming especially when a large number of samples are to be analyzed routinely. In the manometric technique, the respiration rate is measured indirectly from the decrease in oxygen partial pressure in the gaseous phase rather than from the decrease of oxygen activity in the liquid phase. Accordingly, the data obtained may be erroneous in cases where the rate of oxygen absorption from the gas to liquid phase is lower than the oxygen uptake rate of the cells (321), i.e., when the physical oxygen transfer rate becomes the controlling parameter. In still another technique of measurement of endogenous respiration rate, automated versions of which are now in use in some full-scale treatment plants (332,333), a known quantity of biological solids is transferred from the reactor to an air tight and stirred vessel equipped with an oxygen sensor. The DO depletion due to respiration in the initial growing conditions is then measured during an accurately recorded time interval. The respiration rate is determined in mass of oxygen utilized per unit of time per unit of biomass used for the experiment.

Cellular components, which have been used as measures of active biomass, can be classified into two main groups: storage materials (polysaccharides, polyhydroxybutyrate, glycogen, and lipids) and basal materials (nucleic acids, proteins, and enzymes). The cellular contents of the storage materials and nucleic acids vary widely with change in the growth rate and chemical, physical, and physico-chemical factors of the environment (230-234). A constant relationship between dry cell weight and the quantity of any of the components cannot be established for all growth rates since variation of the bacterial density follows a pattern different from that of these cellular constituents. Herbert (230) pointed out that of all the cell constituents, protein content per cell varies the least from cell to cell or from one growth rate to another. This fact has led to the adoption of protein content as a measure of active biomass by some investigators. Use of organic nitrogen content of cultures as an indicator of protein content and viable biomass concentration has been said to be satisfactory in some instances (38). However, the test suffers from the disadvantage that nitrogen content of dead and inactive cells and cell debris as well as non-biological materials would be measured. Nitrogen content higher than normal would be measured when excess and inert nitrogen containing compounds accumulate in cells under carbon limitation or other limiting conditions in the environment.

Apart from the theoretical reasons, the determination of the concentration of any cellular component is time consuming and in some instances involves the use of complicated procedures and equipment. Measurement of proteins, RNA, DNA, or respiration rate was not preferred in this research in view of the theoretical uncertainties as well as the labor and equipment requirements for daily analysis of a large number of samples.

On Enzyme Content as a Measure of Active Biomass. There exist a number of reports indicating that cellular content of an important basal material, the enzymes, bears a linear relationship with the active biomass concentration of the culture. Such observations are compatible with the reports that the activities* of a number of synthetic as well as degradative enzymes such as the "glucose enzymes (glucozymase)" (334, 335), nitratase (336), tetrathionase (337), amino acid decarboxylase (338), and "galactozymaze" (34,128) increase in an "autocatalytic" fashion yielding an S-shaped curve similar to the microbial growth curve. While a strictly theoretical argument in explanation of this phenomenon has not been advanced as yet, at least to the knowledge of this author, it seems logical that enzymes should conform to the same time-concentration relationship as that describing the growth of a cell or population of cells since the latter property is only an expression of the concerted activities of many enzymes. Monod (34) has aptly summarized the situation with the following statement:

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*Enzyme activity has been defined in various ways. Monod (34), Jacob and Monod (25), Spiegelman (128), and others have used this term to mean total content of enzyme protein of a culture. Lamanna and Mallette (13) have defined enzyme activity as the initial velocity of the reaction catalyzed by a given amount of enzyme added to a substrate

> We have seen that the growth of individual cells has been found to be exponential which could be most readily understood as resulting from an inherently autocatalytic growth of each of the cell's population of specific molecules . . . increase of each "strain" of the cell's population of specific enzymes follows autocatalytic function.

The word "autocatalytic" was not used to connote "self-reproducing." Curve 1 of Figure 20 shows the increase in activity of the galactose enzymes in batch culture to be following the typical course of bacterial growth. As mentioned above, other enzymes are known to exhibit similar behavior.

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(continued)

solutions of given concentration. Frequently enzyme activity is expressed as the micromoles of substrate transformed or micromoles of product formed per minute per microgram of enzyme or milliliter of enzyme solution (340). Sometimes activity is expressed in units which are defined differently. Rose (339) defined one unit as the amount of enzyme which produces one micromole of a reaction product per minute under a given standard assay condition. Specific activity may mean the number of units per milligram of protein. Whatever the definition, activity is proportional to the quantity of protein fraction (apoenzyme) of the enzyme.

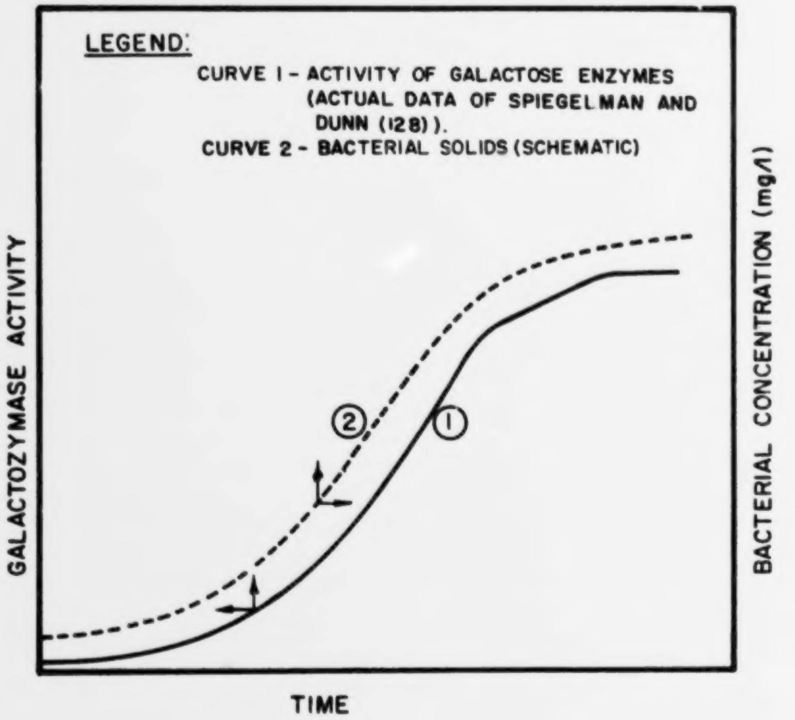


FIGURE 20. SCHEMATIC COMPARISON BETWEEN TIME COURSE OF INCREASE IN THE ACTIVITIES OF GALACTOSE ENZYMES AND BACTERIAL SOLIDS CONCENTRATIONS.

It may be argued that enzymatic activity may not follow a mathematical function similar to that of growth in the stationary and declining phase. However, available evidence does not provide much corroboration. From the results of their studies with starved bacterial culture, Postgate and Hunter (42) have concluded:

> In our experiments "glycerol dehydrogenase" and "glycerol dehydrogenase" activities declined parallel with viability. This parallelism could imply that the relevant enzymic activities remained unchanged until the organism died, when they became negligibly small.

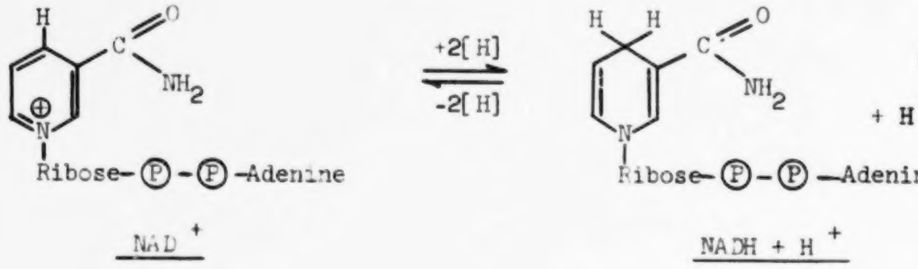
An important conclusion which may be drawn from the above observations is that enzyme content is directly proportional to the quantity of biochemically active cell mass at all specific growth rates, although there do not exist a very large number of reported studies in support of this statement. Apart from this indirect conclusion of linear relationship on the basis of parallelism between growth and enzyme activity, direct observations of such relationships have also been reported. Hershey and Bronfenbrennar (341) observed that enzymatic activity of bacterial protoplasm per unit of dry weight was the same during the phase of adjustment and exponential growth. Monod, _et al_. (342) and Cohn (343) have shown that the ratio of β-galactosidase synthesized to the amount of new growth within the same time (defined as the _differential rate of synthesis_) remained constant during the growth cycle. Benzer (208) made a series of observations similar to the above authors. Furthermore, since formation of active biomass is a result of enzymic activity, the constant of proportionality between them can be expected to remain the same in batch or continuous cultivation thus allowing the usage of the batch calibration in interpreting the enzymatic activity in continuous cultures in terms of microbial activity.

Critical considerations of the available techniques for measuring active organism concentration, in line with the above discussion and in terms of the reliability and simplicity of the test, led to the selection of an enzymatic technique for the determination of active biomass concentrations in this research. Determination of minute quantities of the enzymes is based on simple and sensitive analytical techniques. Photometric analyses are the most commonly used techniques due to the extremely small quantities of enzymes present in the sample.

## On the Optical Techniques of Enzyme Assay

Ultraviolet Spectrophotometry. The optical test for enzyme activity, which has proved to be of great importance in biological research, was introduced by Warburg (344). Later Bergmeyer (345) showed that this method can be applied to aerobic and anaerobic mixed cultures such as those encountered during stabilization of organic wastes.

The test makes use of the light absorbing properties of the hydrogen-transferring enzymes which utilize as their coenzymes, dinucleotides (nicotinamide adenine dinucleotide ($NAD^+$)) one of whose bases is the pyridine derivative of nicotinamide. The function of the aromatic pyridine ring is the reversible uptake of hydrogen. Upon accepting hydrogen, the pyridine ring of $NAD^+$ is reduced to the dihydro form having a broad absorption maximum at 340 mμ in contrast with the pyridine system which does not absorb at that wavelength (30,344). The absorption peak of NADPH occurs at 366 mμ (240).



(127)

Recognizing this property of the pyridine nucleotides, Warburg (344) had recommended measurement of the increase in extinction, ΔA, per unit time at the proper wavelength for determination of the enzyme activity. Light absorption follows Beer's law and the quantity of enzyme can be determined from the following simple equation:

$$\mu\text{moles of dehydrogenase} = \frac{\Delta A_{NADH} \text{ or } \Delta A_{NADPH}}{e \cdot d} \qquad (128)$$

where $A_{NADH}$ and $A_{NADPH}$ are the absorbances at 340 mμ and 366 mμ, respectively

e is the molar absorbancy coefficient, and

d is the light path.

Kotzé (240) has discussed at length the methods of determination of dehydrogenases and various other enzymes in which the unique light absorbing properties of the pyridine system are utilized. For enzymes which are not oxidoreductases, the procedure calls for selecting an appropriate sequence of enzyme reactions in which the enzyme of interest is a participant along with an "auxiliary" dehydrogenase with $NAD^+$ or $NADP^+$. The auxiliary dehydrogenase and substrates are added in excess to the reaction mixture so that the enzyme to be assayed controls the formation of NADH or NADPH, the extinction of which then becomes a measure of the activity of the given enzyme.

Visible Spectrophotometry. Visible spectrophotometric techniques involving the use of aromatic compounds as hydrogen acceptor have been developed taking advantage of the reducing properties of NADH and NADPH. Apart from color formation, this technique has the added advantage of measuring both types of pyridine dinucleotides at the same time. Methylene blue has been used as hydrogen acceptor for the dehydrogenases and as an indicator of bacterial activity in milk sanitation for many years. If a suspension of cells respiring in the absence of oxygen contains methylene blue, the dye acts as a hydrogen acceptor for the dehydrogenases of cells and in so doing the methylene blue is reduced to a colorless leuco-form and the extent of reduction as measured by the extent of discoloration is a measure of the dehydrogenase activity (13,52). The obvious disadvantage with this dye is that the colorimetric measurement of the intensity of the dye solution is an indirect measure of the dehydrogenase activity, and most importantly, the blue color tends to return on the slightest exposure to atmospheric oxygen during spectrophotometric measurement thus jeopardizing any accuracy of the data.

The tetrazolium salts first synthesized by Pechman and Runge (346) have the advantageous characteristic, unlike methylene blue and other indicator dyes, that they are one of few organic compounds which are colored in the reduced state. Kuhn and Jerchel (347), while working on an improved procedure for the synthesis of tetrazolium salts, called attention to the fact that dilute solutions of triphenyltetrazolium chloride (colorless) stained yeast, garden cress, and bacteria due to the reduction of the dilute colorless solution to a red compound tetraphenylformozan. Mattson, et al. (348) and Jensen, et al. (349) recognized that the colorless form of the tetrazolium salt is reduced through the catalytic action of the pyridine nucleotides of dehydrogenase, since the redox potentials of most of these enzyme systems fall below (-) 0.08 volts, the redox potential of 2,3,5-triphenyltetrazolium chloride (TTC) at pH 7.0 as measured by Jerchel and Möhle (350). The potentials of the two redox systems, E-NADH/E-NAD$^+$+H$^+$ and TTC/TF, are such that a spontaneous oxidation-reduction reaction is possible. This latter aspect will be discussed in more detail in the following sections.

Jensen, et al. (349) reported that malic dehydrogenase, β-hydroxybutyric dehydrogenase, lactic acid dehydrogenase, glucose dehydrogenase, and dehydrogenases of plant seeds and embryos could reduce TTC. Kuhn and Jerchel (347) found that glucose dehydrogenases of yeast and bacteria and dehydrogenases of plants reduced the tetrazolium salt. Mattson, et al. (348) observed that TTC stained viable seeds and plant tissues red as a result of reduction by the dehydrogenase system present.

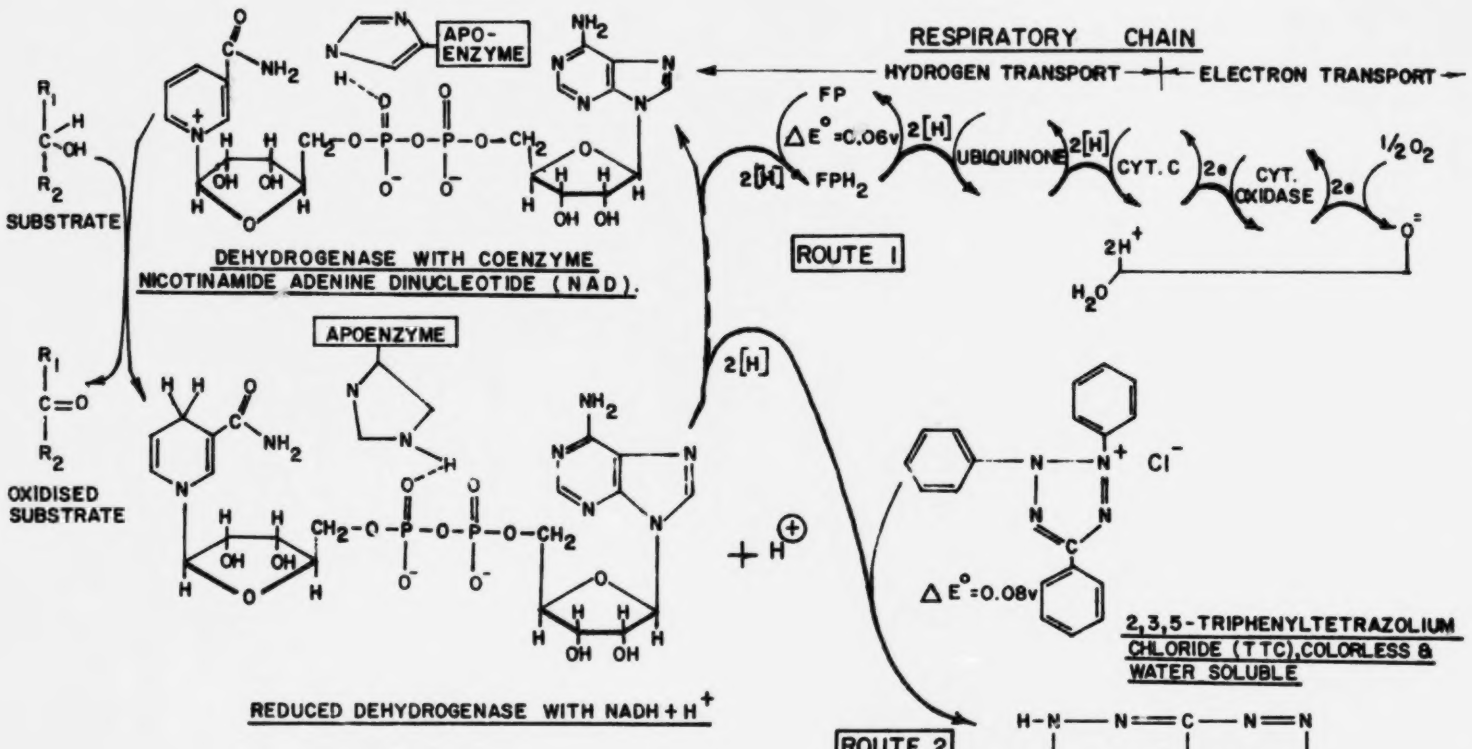
## On the Dehydrogenase Test for Measuring Biochemically Active Biomass Concentration

The versatility of the tetrazolium salt as a hydrogen acceptor for a large number of dehydrogenases in yielding bright red colored solutions for spectrophotometric measurement, and the spontaneity and simplicity of the coupled oxidation-reduction reaction led Lenhard (351) and Bucksteeg and Thiele (352) to adopt dehydrogenase activity, as assayed by the reduction of TTC, to be a measure of the "general biological activity." The authors have reported in a series of several papers (353-362) that the dehydrogenase activity, as measured by the amount TTC reduction gave satisfactory measures of biological activities in sewage, sludge, bottom deposits, and treatment plant effluents. Ford, et al. (363) concluded as a result of laboratory and field investigations in existing waste treatment plants that TTC-measured dehydrogenase activity represents "true sludge activity" and bears a direct relationship with the microbial respiration rate and plant loading factors. An advantage of the use of TTC is due to the fact that growth inhibitors and toxic substances do not interfere with TTC reduction by available dehydrogenases but merely slow down the reduction reaction (364). The latter effect has formed the basis for use of the dehydrogenase test as a criterion for the determination of toxic effects in biological purification systems (360).

## Thermodynamic and Biochemical Bases of the Dehydrogenase Test

Numerous efforts have been expended by the proponents of the dehydrogenase test to develop the test techniques; however, little has appeared concerning discussions on the theoretical basis of the test and its thermodynamic and physiological limitations.

In aerobic biological cultures, oxidation (regeneration) of the reduced nicotinamide dinucleotides ($NADH_2$ or $NADPH_2$) of many dehydrogenases is accomplished by the enzymes (flavins, quinones, and cytochromes) of the respiratory chain. The oxidation is carried out by a series of coupled redox systems (see Figure 21) with oxygen as the final electron acceptor. Although dehydrogenases do not involve oxygen directly, they act upon it indirectly through the flavoproteins and cytochromes since the half-cell potentials of the sequence of redox systems along


RESPIRATORY CHAIN
HYDROGEN TRANSPORT
ELECTRON TRANSPORT
FP
$\Delta E^\circ = 0.06v$
2[H]
$FPH_2$
UBIQUINONE
CYT. C
2e
CYT. OXIDASE
$1/2 O_2$
$O^=$
$2H^+$
$H_2O$
ROUTE 1
ROUTE 2
APO-ENZYME
APOENZYME
SUBSTRATE
OXIDISED SUBSTRATE
DEHYDROGENASE WITH COENZYME NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD).
REDUCED DEHYDROGENASE WITH NADH + $H^+$
$+ H^{\oplus}$
$\Delta E^\circ = 0.08v$
2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE (TTC), COLORLESS & WATER SOLUBLE
TRIPHENYLFORMOZAN (TF) RED, WATER INSOLUBLE
$+ H^+Cl^-$

DESIRABLE CONDITIONS FOR TTC REDUCTION

1. $E(TTC \rightarrow TF) < E(FP \rightarrow FPH_2)$
2. $E(ENZYME\text{-}NADH \rightarrow ENZYME\text{-}NAD) > E(TTC \rightarrow TF)$

OXIDATION OF NADH OR NADPH IS MADE TO PROCEED VIA ROUTE 2 BY ELIMINATING MOLECULAR OXYGEN FROM THE REACTION MIXTURE AND BY PROVIDING LARGE EXCESS CONCENTRATION OF TTC.

FIGURE 21. ALTERNATIVE ROUTES OF OXIDATION OF DEHYDROGENASES IN THE PRESENCE OF TTC.

this indirect path of hydrogen or electron transfer are such that the coupled oxidation-reduction reactions proceed spontaneously (30). In the dehydrogenase test, the redox system TTC/TF is placed parallel to and in competition with the respiratory enzymes. Being the first oxidative system in the respiratory chain, the flavoproteins are in direct competition with the TTC for accepting hydrogens from $NADH_2$ or $NADPH_2$. If the standard half-cell redox potentials (E°) at pH 7.0 of the half-cells $FP \rightarrow FPH_2$, E°=0.06 volts (30) and $TTC \rightarrow TF$, E°= 0.08 volts (350), are compared, it is evident that hydrogen transfer through the flavoproteins and the other enzymes in the series is more spontaneous and preferable than reduction of the TTC. However, half-cell potentials* (E) rather than standard half-cell potentials (E°) in non-standard conditions should be compared.

Despite this apparent thermodynamic disadvantage, the reaction conditions can be adjusted by manipulation of the pH, temperature, and concentration of TTC (which is the oxidized form of the half-cell TTC/TF), so that the half-cell potential of TTC/TF (as calculated from the Nernst equation*) favors the reduction of TTC. An important condition, apart from competition with the flavoproteins, is that the dehydrogenase system must have a redox potential higher than that of the TTC/TF system (e.g., more than 0.08 volts under standard conditions of temperature and

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*E is given by the Nernst equation:

$$E = E^\circ - 2.3 \frac{RT}{nF} \log \frac{[\text{oxidized form}]}{[\text{reduced form}]} \qquad (129)$$

where F = 96,500 coulombs/equivalent
R = 8.314 Joules $deg^{-1}$ $mole^{-1}$
n = number of electrons transferred

pressure) in order for it to be oxidized by TTC. Thus, the glutamic dehydrogenase system, having a redox potential of 0.03 volts, cannot reduce TTC (349). The two important conditions for reduction of TTC can now be summarized as follows:

For formation of tetraformozan and red color,

$$E/_{TTC \rightarrow TF} \leqq E/_{FP \rightarrow FPH_2} \qquad (130)$$

$$E/_{\text{apoenzyme : } NADH_2 \rightarrow \text{apoenzyme : } NAD} > E_{TF \rightarrow TTC} \qquad (131)$$

The formation of tetraformozan depends on the accessibility of the various dehydrogenases to TTC. Whether transport of TTC into the cell is accomplished by passive diffusion or via any permease type mediation remains a moot question. It is only known that the reagent can permeate into different parts of a cell given sufficient time. Since TTC was observed to be reduced by many of the enzymes of the tricarboxylic cycle (349) resident within the mitochondria (30), it is apparent that the reagent can be transported across the mitochondial membrane even though it is impermeable to many enzymes. However, penetration into the mitochondria does not guarantee formation of TF, since the respiratory enzymes which are also located in mitochondria may interfere with the reduction of TTC. Many of the dehydrogenases outside the mitochondria cannot pass through the mitochondrial membrane and, in their reduced form, they do not relinquish hydrogen through the respiratory chain. The dehydrogenases of anaerobic metabolism and the NADP linked dehydrogenases used in the synthetic pathways are examples. Some of these enzymes are located on the cytoplasmic membrane or the soluble portion of the cytoplasm (365) and are more accessible than the mitochondrial dehydrogenases. Reduced forms of these enzymes can easily relinquish hydrogens to TTC provided Equation 131 is satisfied. These reactions are free from interferences by the respiratory enzymes. Evidence of extra-mitochondrial dehydrogenases having been oxidized by TTC is furnished from the studies of Malatyan and Biryuzoza (366) who observed deposition of formozan on the dehydrogenase surfaces.

It can be concluded from the above discussion that some reduction of TTC and formation of color would take place despite any transport limitation and interference by oxygen simply because of the capability of TTC to "diffuse" into different parts of the cell and react with extra-mitochondrial dehydrogenases. Nevertheless, the intensity of the color may not be reproducible and consistent with oxidizable dehydrogenases when such limitations and interferences exist.

Under conditions of transport limitation, the measured activity in terms of TF formation may be conditioned by the accessibility of the enzymes instead of being determined by the amounts of enzymes present. To alleviate this problem, the standard techniques of enzyme assay involve disruption of the cells and release of the enzymes by ultrasonic or mechanical disintegration (340,52) or explosive decompression[*] (367, 368). The last technique, which has been claimed to be advantageous over the other two, involves pressurizing the cell suspension with compressed nitrogen during a pressure cycle and subsequently rupturing the cell by explosive decompression. The percentage of cells ruptured is reproducible from test to test and any error due to variation of this factor is avoided. Cell rupture results in an increase of measured activity (52), increased accuracy of optical measurement, and elimination of the possibility of TTC-transport limitation to have any bearing on the results.

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[*] An apparatus called the "Cell Disruption Bomb" is now commercially available (368).

The test procedure proposed by Lenhard and others provides a test environment almost ideal for unhampered growth and multiplication of the organisms in the sample during the reaction period. This would introduce a positive error due to inclusion in the measurement of the activities of new enzymes formed in existing cells of the sample as well as the enzymes of the progeny. Though not recommended by the above authors, in standard enzyme assay procedures, growth is prevented by centrifuging the cell suspension at two degrees Centigrade, suspending the pellet in a buffer solution, disintegrating the cells at temperatures not exceeding eight degrees Centigrade, and finally recovering the enzyme suspension by separating it from the cell debris by centrifugation (240).

To prevent the occurrence of any cell division, Lenhard (359) and Ford, et al. (3[illegible]) have recommended a reaction time of 15 minutes since this duration is shorter than most generation times encountered. It is to be noted that, regardless of the generation time, some of the cells would divide during this 15 minute period as the process of cell division would have been conceivably initiated in these cells before being drawn into the sample.

Lastly, it should be mentioned that the measurable quantities of dehydrogenase per milliliter of culture volume are a rather unique quantity, the magnitude of which is dependent on the physical, chemical, and biological factors of the culture environment as well as the details of the techniques of measurement. Any correlation established for a particular system is not ordinarily expected to be valid in other cases and should be used with caution and scientific judgment.

Design of the Dehydrogenase Test Procedure. It follows from the Nernst equation that a lower value of $E/_{TTC\rightarrow TF}$, which is desirable according to previously stated information of Conditions 130 and 131 would result due to the increase of temperature and the molar concentration of TTC. A lower value of the half-cell potential would mean increased rate of production of formozan as observed by Lenhard (359) and Ford, *et al*. (363). A temperature of 37°C is suitable since higher temperatures do not show any appreciable improvement in color production.

As would be predicted from the Nernst equation, the quantity of tetraformozan produced increased as TTC concentration was increased from 0.01 percent to 0.05 percent in Lenhard's experiments (359). A selection of TTC concentration of 0.01 percent by Lenhard appears to be unjustified in view of the increased value of $E/_{TTC\rightarrow TF}$ and creation of a better thermodynamic condition for interference by oxygen. A 0.2 percent solution of TTC as recommended by Ford, *et al*. (363) was used in this research. Furthermore, increases of TTC concentration over this value do not cause any appreciable decrease in the value of $E/_{TTC\rightarrow TF}$.

The redox potential of the TTC→TF system decreases with an increase in pH and, in highly alkaline solutions, non enzymatic compounds may also reduce TTC thereby causing interference with enzymatic reduction. For example, Mattson, *et al*. (348) have pointed out that reducing sugars become potent reducers of TTC at pH 11 or higher. Therefore, color intensity would increase with an increase in pH and this property would have been desirable for the sake of accurate measurement, shorter reaction time, etc. had the increase been entirely attributable to enzymatic reduction of TTC at higher pH. The desire to have an increased rate of formozan production has to be balanced with the need for elimination of non-enzymatic reduction of TTC and pH of 8.4 has been found to be suitable for various types of cultures (359). Based on experience with laboratory mixed cultures and biological sludge from waste treatment plants, Ford, *et al*. (363) suggested that "it is mandatory to stay within the pH seven to pH nine limits."

It follows from Figure 21 that the concentration of tetraformozan produced is dependent on the concentration of reduced dehydrogenase in the sample. In order to obtain a correct measure of the total dehydrogenase content of the sample, it is necessary that any unreduced dehydrogenase in the sample be converted to the reduced form since only in this form can the enzymes reduce the tetrazolium salt. Because all samples may not contain substrates in sufficient concentration, addition of extraneous substrate in large concentration would ensure the conversion of the unreduced form of the coenzymes of dehydrogenases to the reduced form at the maximum velocity of the corresponding enzymatic step. The rate of formation of tetraformozan would thus be controlled by the concentration of the dehydrogenases present and not by the concentration of the substrate. The properties of the substrate should be such that either it or its catabolites can become substrates for the dehydrogenases. No substrate can satisfy this requirement better than glucose which is added in a large concentration to the sample. Lactate, citrate, succinate, and glutamate, all catabolites of glucose, were reported to be less effective individually than glucose as an added substrate (359).

Two important impediments to the accuracy of the test results are the presence of dissolved and atmospheric oxygen and growth of the microbial population during the test duration. The interference or inhibition by oxygen in lessening the intensity of color and causing formation of non-reproducible quantities of formozan was first observed by Marlar (221). Of the methods tried by Marlar to eliminate the inhibitory effect of oxygen, bubbling of nitrogen through the test samples was most successful in alleviating the difficulties. In this study, it was observed that color intensity increased with increase in bubbling rate of nitrogen up to 0.01-0.02 SCFM beyond which improvement in intensity was imperceptible. Apparently a minimum flow rate of 0.01 SCFM is needed to expel all DO present in the sample and to maintain anaerobic conditions by counteracting the spontaneous tendency of oxygen absorption from the atmosphere. The effect of the nitrogen flow rate could be evidenced very clearly when duplicates from the cell suspension were run at unequal flow rates to produce strikingly different intensities of color.

The simple apparatus shown in Figure 22 proved to be very satisfactory in obtaining an oxygen free atmosphere for running a series of samples under identical nitrogen flow rates and eliminating any variability due to unequal and insufficient nitrogen flow. An important advantage of the nitrogen flow pattern was that the steady stream of pressurized nitrogen flow through the closed system of tubes helped maintain a nitrogen atmosphere above the reaction mixture. An anaerobic atmosphere

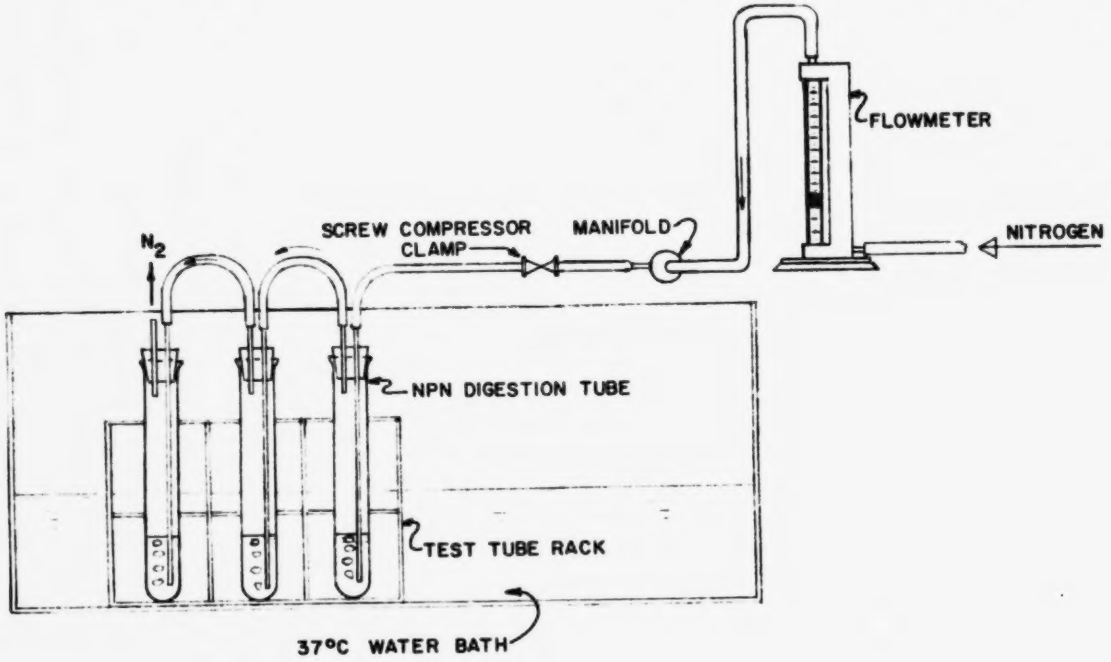
FLOWMETER
NITROGEN
$N_2$
SCREW COMPRESSOR CLAMP
MANIFOLD
NPN DIGESTION TUBE
TEST TUBE RACK
37°C WATER BATH

FIGURE 22. APPARATUS FOR THE DEHYDROGENASE TEST.

both in the liquid and the gaseous phases performed the important function of preventing growth during the incubation period.

In order to improve the color intensity, it was decided to homogenize all samples at a speed of 15,000 rpm for a fixed time interval thereby mechanically disintegrating a fraction of the cell population. Apart from increased production of formozan, disintegration also prevented growth of the fraction of ruptured cell populations.

Since disintegration of cells and the nitrogen atmosphere eliminated the possibility of growth, longer incubation time could be used for dilute samples for production of more intense color. With this technique, selection of short reaction time was no longer necessary to preclude the possibility of cell division.

Due to the provision of large concentrations of TTC and glucose in the reaction mixture, the formation of the colored compound follows a zero-order kinetics and proceeds at the maximum velocity of the enzymatic reduction of TTC. The rate tends to decline after an interval determined by the amount of dehydrogenase initially present. Additional color formation after this interval is but a small percentage of the total color to be formed and the reaction may be stopped after this interval of time without sacrificing any appreciable gain in tetraformozan formation. Unduly long incubation periods are undesirable. The data of Ford, et al. (363) from studies with biological sludges of different physiological characteristics and concentrations indicated that the rate of production of tetraformozan declines rapidly after 60 minutes in the cases examined and, based on this information, a reaction time of 60 minutes was chosen for this research. A choice of an incubation period of 60 minutes is not expected to give rise to bacterial multiplication since the generation times of the bacteria in the chemostat samples were higher than this period in most operating conditions. Thus, cell division would not have occurred in most instances even if a nitrogen atmosphere was not maintained.

It should be emphasized that homogenization of the sample and elimination of oxygen from the reaction mixture are important for dilute cultures since, without these measures, the quantity of tetraformozan production would be less and unrepresentative of the quantity of dehydrogenase present.

It was imperative to perform all tests under similar experimental conditions since dehydrogenase activities in chemostat samples were interpreted in terms of active solids by comparison with calibration curves prepared earlier. Since TTC reduction is a function of temperature, pH, and time of reaction, it was necessary to carry out all tests at the constant pH of 8.4 and temperature of 37°C and to terminate the reactions exactly after 60 minutes in all cases.

A detailed procedure of the dehydrogenase test as used in this research is given in Appendix III.

## On the Establishment of Correlation Curves between Dehydrogenase Activities and Active Biomass Concentration

### Design of the Correlation Experiments.

The dehydrogenase test was developed with the objective that the test was to be used for monitoring active biomass concentrations in the continuous culture runs which were the principal experiments. It was decided to establish correlation curves between dehydrogenase activity and active biomass concentration from the results of several batch experiments to be performed with glucose and galactose as the substrates. Several batch runs were planned with the same sugar, but to be seeded with inoculums obtained at various detention times of the continuous flow reactor. Based on the discussion in Chapter II, it was anticipated that continuous cultures at different detention times might contain different dominant groups of microorganisms and that different dominant populations might not exhibit the same correlation between dehydrogenase activity and active biomass concentration. It was felt that, if several batch runs were made with inoculums derived from continuous cultures maintained at different detention times, then it would be possible to ascertain whether the correlations between dehydrogenase activity and biomass concentration were different for the continuous culture populations of different detention times.

For a given dominant culture it is possible that the relationship between dehydrogenase activity and biomass concentration may be different for different specific growth rates. In order to verify this possibility, two types of sampling and analysis were planned for each batch experiment:

a) samples, referred to as "straight samples," were to be drawn at different points (representing different specific growth rates) of the batch growth, and

b) single sample, to be drawn at a point near the end of the exponential phase, was to be diluted to yield several "diluted samples." Each "straight" or "diluted" sample was to be analyzed for dehydrogenase activity and mass concentration of the dry organisms. Comparison of the correlations between dehydrogenase activities and dry solids concentrations established from the results of the tests with "straight" and "diluted" samples would indicate any effect of specific growth rates on the said correlation.

An assumption made in the development of the aforementioned correlation was that the measured solids concentration in the reactor was equal to the concentration of active biomass. It has been reported that, in batch cultures, dead cells are practically undetectable in the acceleration phase and in the early part of the exponential phase (13,28). Viability, i.e., the ability to reproduce, may decrease in the later part of the exponential phase. However, it is possible that many of the cellular processes continue at the expense of the extracellular substrate which is not completely exhausted at the end of the exponential phase. Thus, although some cells may not be able to reproduce at the later part of the exponential phase, they may be considered active in that some living processes are still continued at the expense of substrate. It was, therefore, assumed that dry solids concentrations of samples drawn from dilute batch cultures before the termination of the exponential phase would not be significantly different from the active solids concentrations.

Procedure for Batch Experiments. The reactor used for the batch runs is shown in Figure 23. In many respects, the design of the batch reactor was similar to that of the continuous flow reactor. The reactor contents were mixed at a fixed speed of 118 rpm, which remained constant for all runs and provided adequate mixing and oxygen transfer. The runs were performed at 20°C by placing the reactor in the constant temperature water bath. The growth of the culture was monitored by the dissolved oxygen depletion curve as recorded by an arrangement essentially similar to that of the continuous culture system and shown in Figure 23.

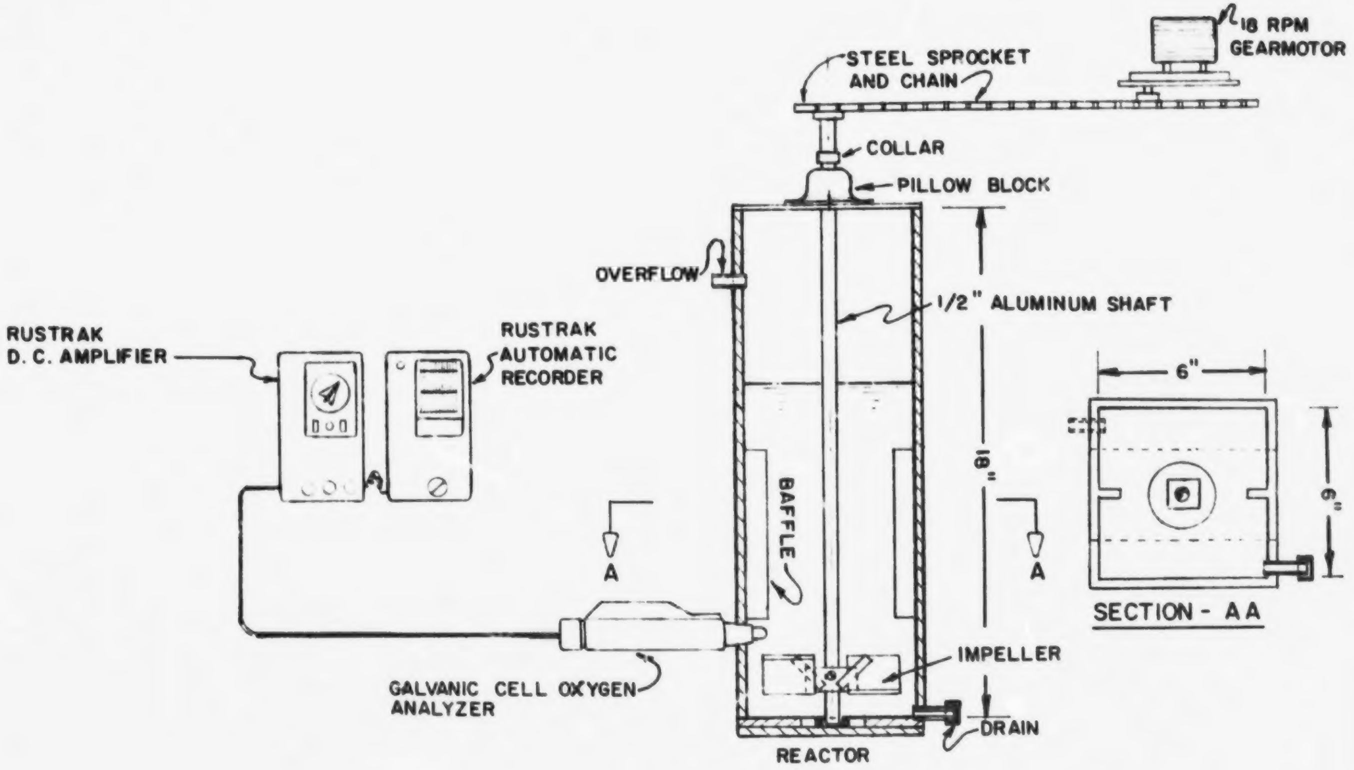
STEEL SPROCKET
AND CHAIN
18 RPM
GEARMOTOR
COLLAR
PILLOW BLOCK
OVERFLOW
1/2" ALUMINUM SHAFT
RUSTRAK
D. C. AMPLIFIER
RUSTRAK
AUTOMATIC
RECORDER
6"
18"
6"
BAFFLE
A
A
SECTION - AA
IMPELLER
GALVANIC CELL OXYGEN
ANALYZER
DRAIN
REACTOR

FIGURE 23. REACTOR SYSTEM FOR GROWING BATCH CULTURES.

The reactor was filled to a volume of five liters with minimal media[*] (see Appendix IV) and using glucose or galactose as the carbon and energy source. The temperature was adjusted to 20°C and the reactor seeded with an inoculum obtained from the continuous reactor operated at a selected dilution rate. Samples were withdrawn for determination of dehydrogenase activity and bacterial solids concentration at intervals selected according to the course of the oxygen depletion curve. Sampling was discontinued after the oxygen profile reached the minimum concentration level at which point the culture entered the retardation phase.

The concentration of dry solids was determined by filtering a known volume of the sample through a millipore filter of pore size of 0.45 μ so that the bacterial cells could be retained on the filter paper. As considerable difficulties were experienced in weighing the filter paper due to moisture, absorption, etc., a control filter method (369, 370)(see Appendix V) was used. The method involved placing two preweighed millipore filters--one upon the other--in the millipore filter holder and filtering the sample through both filters. The bacterial solids were retained entirely by the top or test filter while the bottom or control filter was carried through the analysis in order to control and correct for test variables and possible sources of error. The control filter was subjected to the same procedures as each test filter with the exception of actual solids filtration, thereby serving as an indicator of any changes in the calibration of the weighing balance, changes in filter weights resulting from moisture content changes (humidity variation), desorption of filter extractables, or adsorption and/or absorption of fluid constituents. Detailed procedures for solids determination are presented in Appendix V.

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[*] In a minimal synthetic medium, provisions are made for satisfying only the "minimal" nutritional requirements, such as the essential elements (C,N,P,S,etc.) and a few of the trace elements (K,Na,Ca,Fe,Mg, Mn,Co,Zn,etc.). Thus, a minimal medium is a simple synthetic medium consisting of ammonium salts, phosphates, sulfates, and other mineral salts with the addition of an organic compound as a source of carbon and energy.

Presentation of the Batch Data. The basic data from the various batch runs are presented in tabular form in Appendix VI. Tables 2 and 3 include the dehydrogenase activities (measured with one centimeter light path) and biomass concentration of straight samples from all batch cultures grown on glucose and galactose, respectively. Table 4 includes dehydrogenase activities (measured with one centimeter light path) and biomass concentrations of diluted samples from all batch cultures fed with galactose.

For very dilute cell suspensions, reliable absorbance readings could not be obtained with a one centimeter light path and a longer light path of 10 centimeters was used. Table 5 presents the dehydrogenase activities, as measured with a 10 centimeter light path, and biomass concentrations of straight and diluted samples from all runs using glucose or galactose as the substrate.

Discussion of Batch Culture Data. The dehydrogenase data of some of the batch data were plotted as a logarithmic function of time in Figure 24 to show that enzymatic activity increased exponentially with time as observed by Spiegleman and others (34,128,334-338). Figure 25 is a plot of a typical batch run; the parallel increase of dehydrogenase activity and biomass concentration provided experimental verification of

Table 2. Correlation between Dehydrogenase Activity at One cm Light Path and Concentration of Dry Solids (Active Biomass) Grown on Glucose as Substrate
(Samples at Different Times during Batch Growth)

| Batch No. | Dehydrogenase Activity (O.D. @ 483 mμ and 1 cm Light Path) $\times 10^4$ | Dry Solids Concentration (mg/ℓ) |
|---|---|---|
| 1 | 162 | 5.3 |
| | 470 | 20.0 |
| | 364 | 28.0 |
| | 565 | 37.0 |
| | 536 | 33.0 |
| | 977 | 44.0 |
| | 398 | 52.0 |
| | 659 | 55.0 |
| 2 | 128 | 5.0 |
| | 160 | 6.5 |
| | 264 | 9.0 |
| 3 | 789 | 47.5 |
| | 942 | 61.0 |
| | 1934 | 82.0 |
| 4 | 327 | 18.0 |
| | 388 | 22.0 |
| | 1388 | 68.0 |
| 5 | 269 | 10.5 |
| | 258 | 24.7 |
| | 633 | 38.7 |
| 6 | 44 | 1.0 |
| | 72 | 6.5 |
| | 174 | 10.7 |
| | 258 | 20.7 |
| | 1173 | 64.0 |
| | 2048 | 110.0 |
| | 1581 | 90.0 |
| 7 | 593 | 36.0 |
| | 1773 | 88.0 |
| | 2427 | 138.4 |
| | 2573 | 162.0 |
| | 2226 | 132.0 |
| | 2253 | 122.0 |
| | 2091 | 146.0 |

Table 3. Correlation between Dehydrogenase Activity at One cm Light Path and Concentration of Dry Solids (Active Biomass) Grown on Galactose as Substrate
(Samples at Different Times during Batch Growth)

| Batch No. | Dehydrogenase Activity (O.D. @ 483 mμ and 1 cm Light Path) x $10^4$ | Dry Solids Concentration (mg/ℓ) |
|---|---|---|
| 1 | 171 | 8.5 |
| | 198 | 6.0 |
| | 237 | 10.0 |
| | 223 | 12.0 |
| | 315 | 13.0 |
| | 463 | 22.0 |
| | 872 | 36.0 |
| | 932 | 47.0 |
| 2 | 126 | 5.0 |
| | 141 | 9.5 |
| | 155 | 12.0 |
| | 214 | 40.1 |
| | 600 | 33.0 |
| 4 | 2417 | 133.3 |
| 5 | 92 | 13.0 |
| | 119 | 15.0 |
| | 103 | 14.0 |
| | 244 | 28.0 |
| | 320 | 40.0 |
| | 458 | 44.0 |
| | 633 | 46.0 |
| | 745 | 52.0 |
| | 2330 | 118.0 |

Table 4. Correlation between Dehydrogenase Activity at One cm Light Path and Concentration of Dry Solids (Active Biomass) Grown on Galactose as Substrate (Samples at One Point of Exponential Growth; Dehydrogenase Activity Determined on Straight Sample and its Various Dilutions)

| Batch No. | Dehydrogenase Activity of Straight/Diluted Sample (O.D. @ 483 mμ and 1 cm Light Path) x $10^4$ | Dilution Factor | Dry Solids Concentration (mg/ℓ) |
|---|---|---|---|
| 1 | 738 | 0.40 | 29.2 |
| | 487 | 0.20 | 14.6 |
| | 115 | 0.05 | 3.65 |
| 4 | 2417 | 1.000 | 133.3 |
| | 1789 | 0.800 | 106.7 |
| | 1033 | 0.600 | 80.0 |
| | 760 | 0.400 | 53.3 |
| | 232 | 0.200 | 26.7 |
| 5 | 2330 | 1.000 | 118.0 |
| | 1902 | 0.800 | 94.4 |
| | 790 | 0.600 | 70.8 |
| | 732 | 0.400 | 47.2 |
| | 420 | 0.200 | 23.6 |
| | 209 | 0.100 | 11.8 |

Table 5. Correlation between Dehydrogenase Activity at 10 cm Light Path and Concentration of Dry Solids (Active Biomass)

| Batch No. | Substrate | Dehydrogenase Activity (O.D. @ 483 $m\mu$ & 10 cm Light Path) $\times 10^4$ | Dry Solids Concentration (mg/ℓ) | Dilution Factor | Type of Sample |
|---|---|---|---|---|---|
| 4 | Galactose | 492 | 6.0 | 1 | Straight samples from different points of batch growth |
| | " | 685 | 7.0 | 1 | |
| | " | 1276 | 31.0 | 1 | |
| | " | 2259 | 32.5 | 1 | |
| | " | 3472 | 36.0 | 1 | |
| | " | 3780 | 37.0 | 1 | |
| | " | 3360 | 55.0 | 1 | |
| 5 | Galactose | 506 | 2.0 | 1 | ↓ |
| | " | 3835 | 44.0 | 1 | |
| 7 | Glucose | 50 | 1.0 | 1 | |
| | " | 1201 | 13.5 | 1 | |
| 4 | Galactose | 614 | 6.7 | 0.05 | Dilutions of straight sample from a point in batch growth |
| | " | 964 | 13.3 | 0.10 | |
| 5 | Galactose | 585 | 5.9 | 0.05 | |
| | " | 1555 | 11.8 | 0.10 | |
| 7 | Glucose | 655 | 6.3 | 0.05 | |
| | " | 1297 | 12.6 | 0.10 | |
| | " | 2262 | 25.2 | 0.20 | |

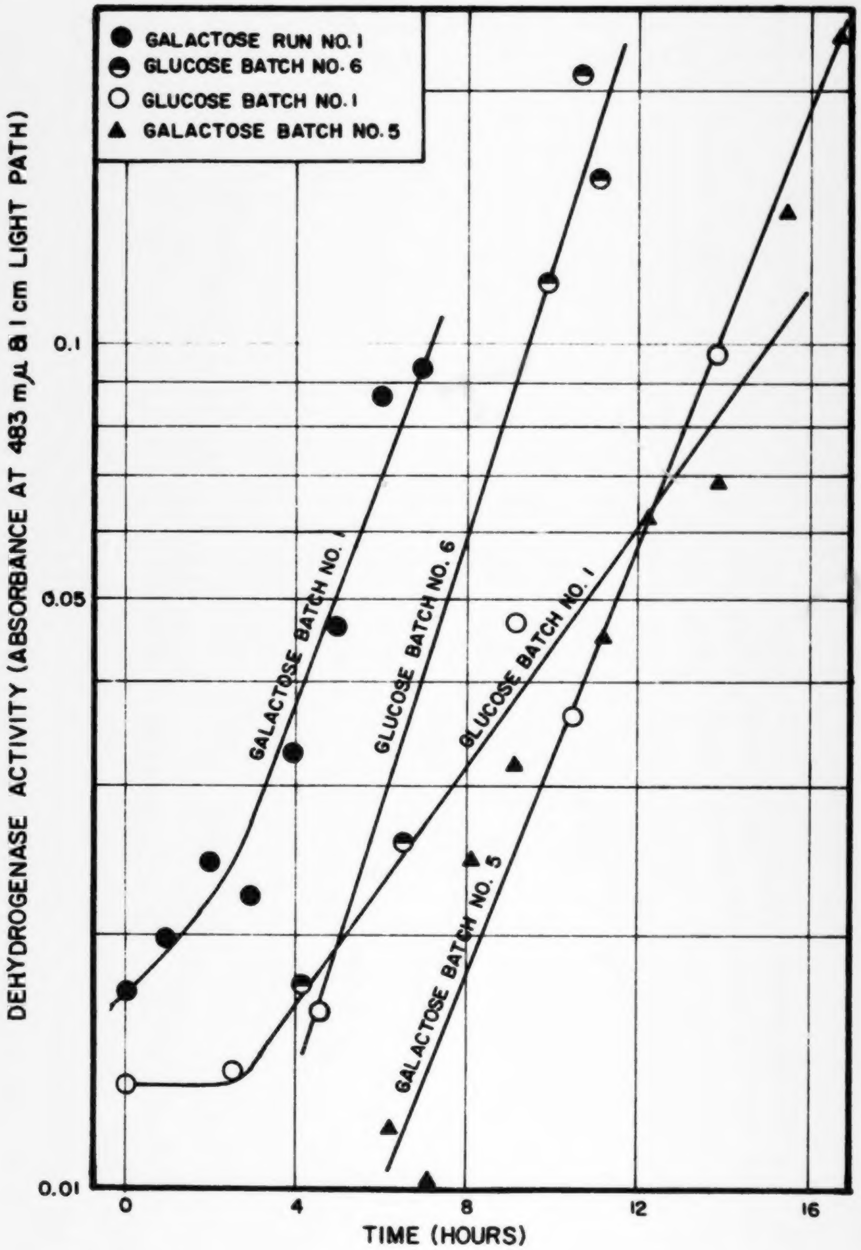


FIGURE 24. DEHYDROGENASE ACTIVITY AS A FUNCTION OF TIME IN BATCH CULTURES.

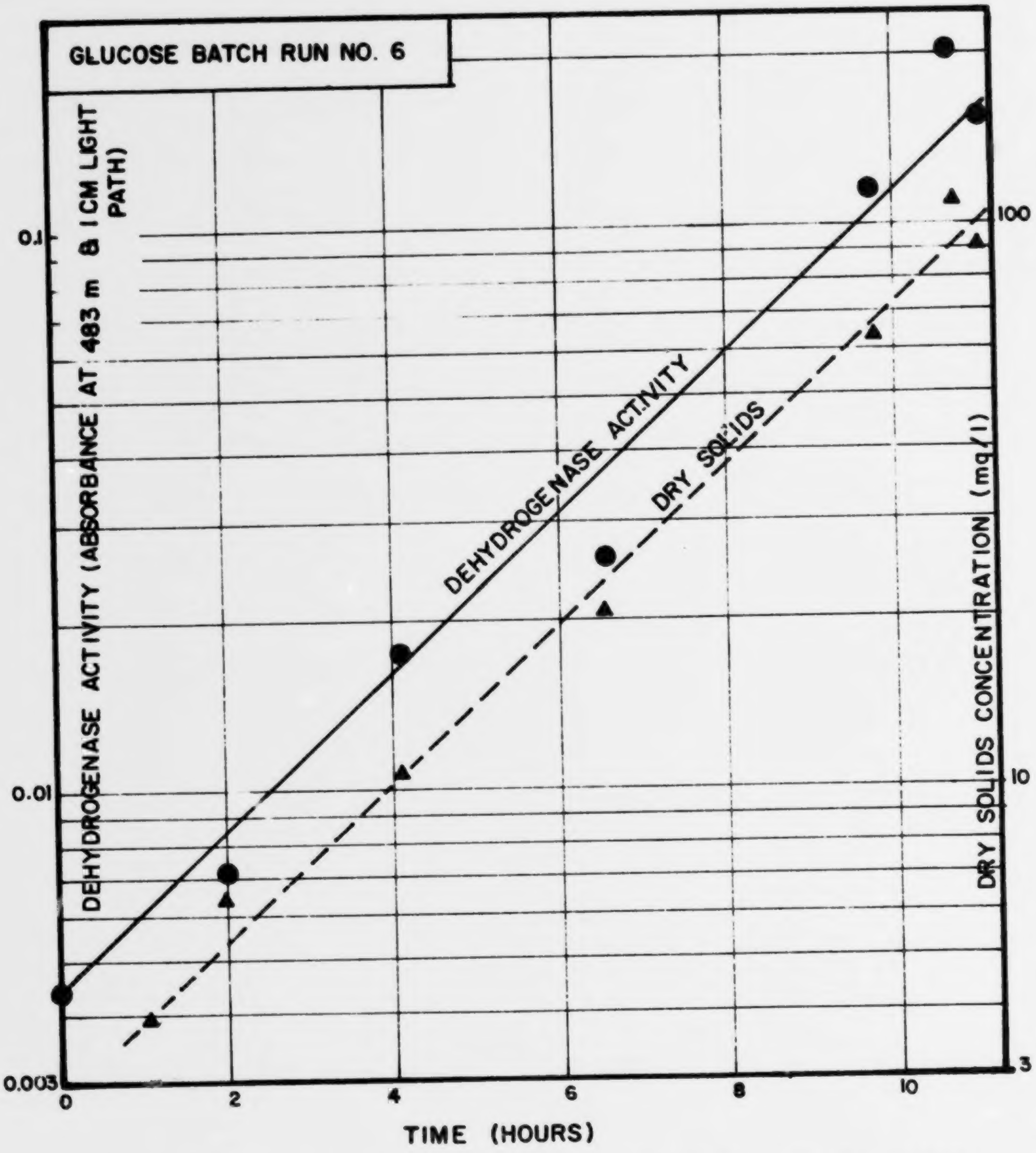


FIGURE 25. TIME COURSE OF INCREASE OF DEHYDROGENASE ACTIVITY & ACTIVE BIOMASS CONCENTRATION IN BATCH CULTURES.

the premise that the constant of proportionality between dehydrogenase activity and biomass concentration remained the same at all growth rates.

The data of Tables 2 and 3 are plotted on Figures 26 and 27, respectively. The linear regression equations describing the plots of these data, and the high correlation coefficients indicated almost perfect stochastic linear dependence of active biomass concentration on dehydrogenase activity. The linear dependence of biomass concentration on dehydrogenase activity, as predictable from the parallelism of these parameters shown in Figure 25, also held statistically. Thus, dehydrogenase activity was a linear function of biomass concentration when glucose or galactose served as the substrate; the relationship remained the same at all growth rates of the acceleration and exponential phase. The following relationships between dehydrogenase activity (as measured with a one centimeter light path) and biomass concentration were obtained for the glucose and galactose fed cultures.

For glucose grown cultures,

$$X^o = 1.9 + 560\ A_1 \tag{132}$$

(Correlation coefficient = 0.972)

For galactose grown cultures,

$$X^o = 6.8 + 498\ A_1 \tag{133}$$

(Correlation coefficient = 0.957)

where $X^o$ = active biomass concentration in mg/$\ell$

$A_1$ = absorbance by triphenylformozan in one centimeter light path.

It should be noted that the above linear correlations were established from analysis of data on straight samples.

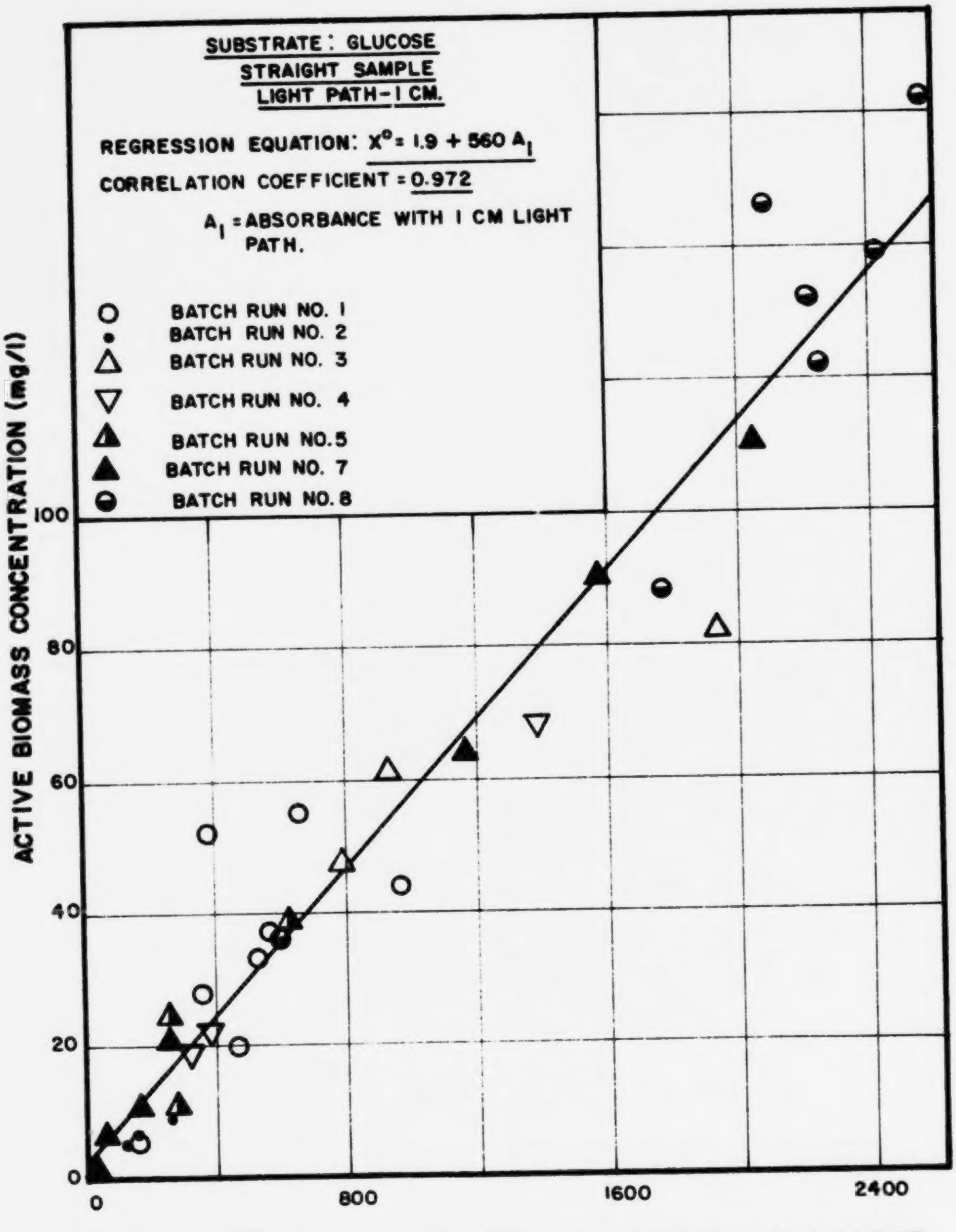


FIGURE 26. CORRELATION BETWEEN DEHYDROGENASE ACTIVITY AND CONCENTRATION OF ACTIVE BIOMASS IN GLUCOSE GROWN BATCH CULTURES.

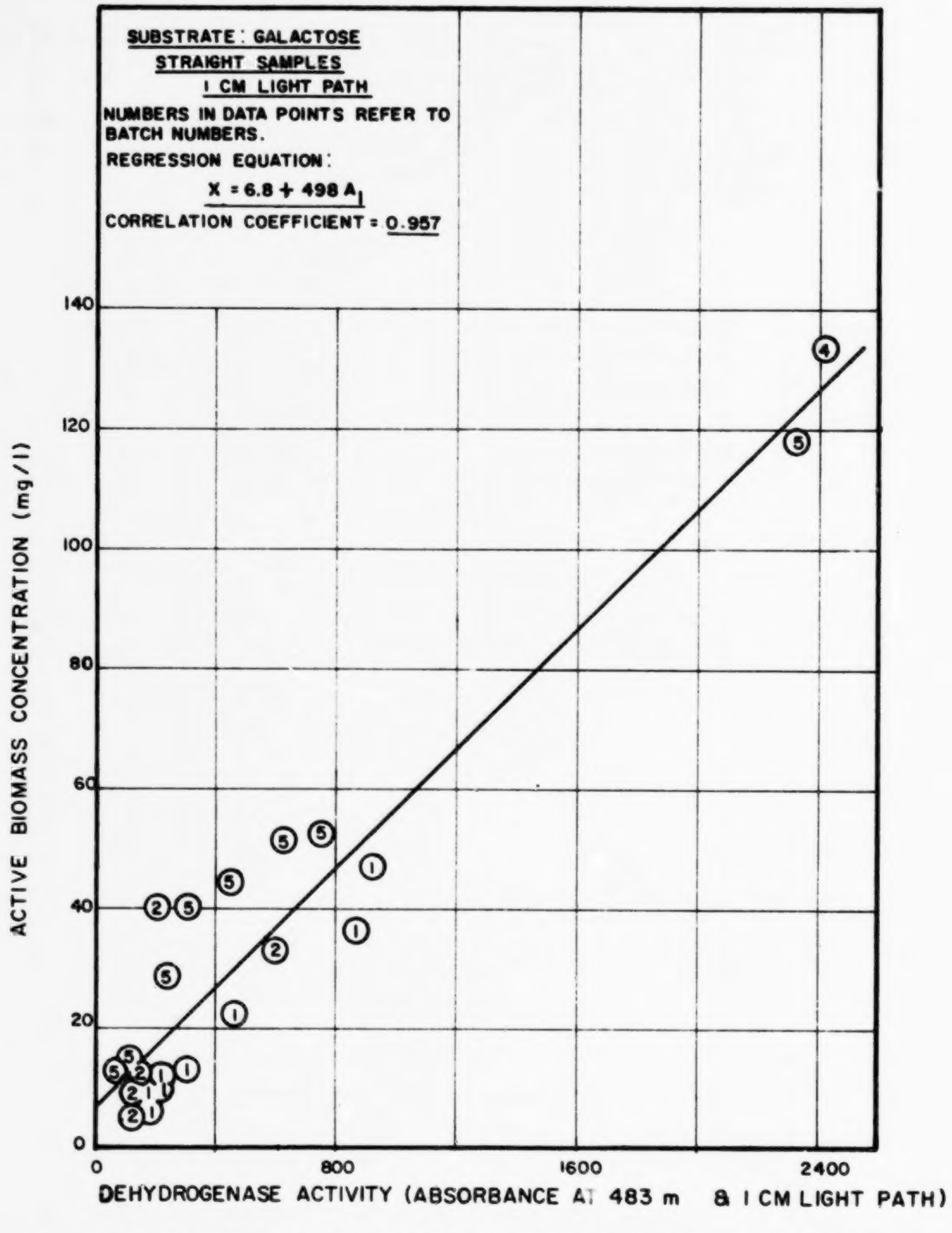


FIGURE 27. CORRELATION BETWEEN DEHYDROGENASE ACTIVITY AND CONCENTRATION OF ACTIVE BIOMASS IN GALACTOSE GROWN BATCH CULTURES.

Although cell yield was not of any importance in establishing the foregoing correlations, it was observed that the growth yield coefficients on glucose and galactose in batch cultures were 0.40 and 0.31, respectively. These results may be used to compare with the growth yields on these substrates when fed to continuous cultures.

The data of Table 4 were plotted on Figure 28. The regression line and the correlation coefficient of Figure 28 indicated that dehydrogenase activities of the diluted samples of galactose grown cultures were also linearly related to biomass concentration in accordance with the following equation:

For galactose grown culture,

$$X^{o} = 6.3 + 520\ A_1 \qquad (134)$$

(Correlation coefficient = 0.958)

While both Equations 133 and 134 apply to galactose grown cultures, it should be recognized that the former relationship was based on straight samples drawn at various growth rates, whereas the latter equation was based on dilutions of single samples drawn at a particular growth rate near the end of the exponential phase. A statistical test of the hypothesis that the two regression lines represented by Equations 133 and 134 were identical (371) showed that there were no significant differences between the slopes and intercepts of these lines at the five percent level of significance. Statistically, therefore, Equations 133 and 134 were estimates of the same linear relationship. This meant that the same linear relationship between dehydrogenase activity and biomass concentration was valid for all growth rates. Furthermore, it appears that

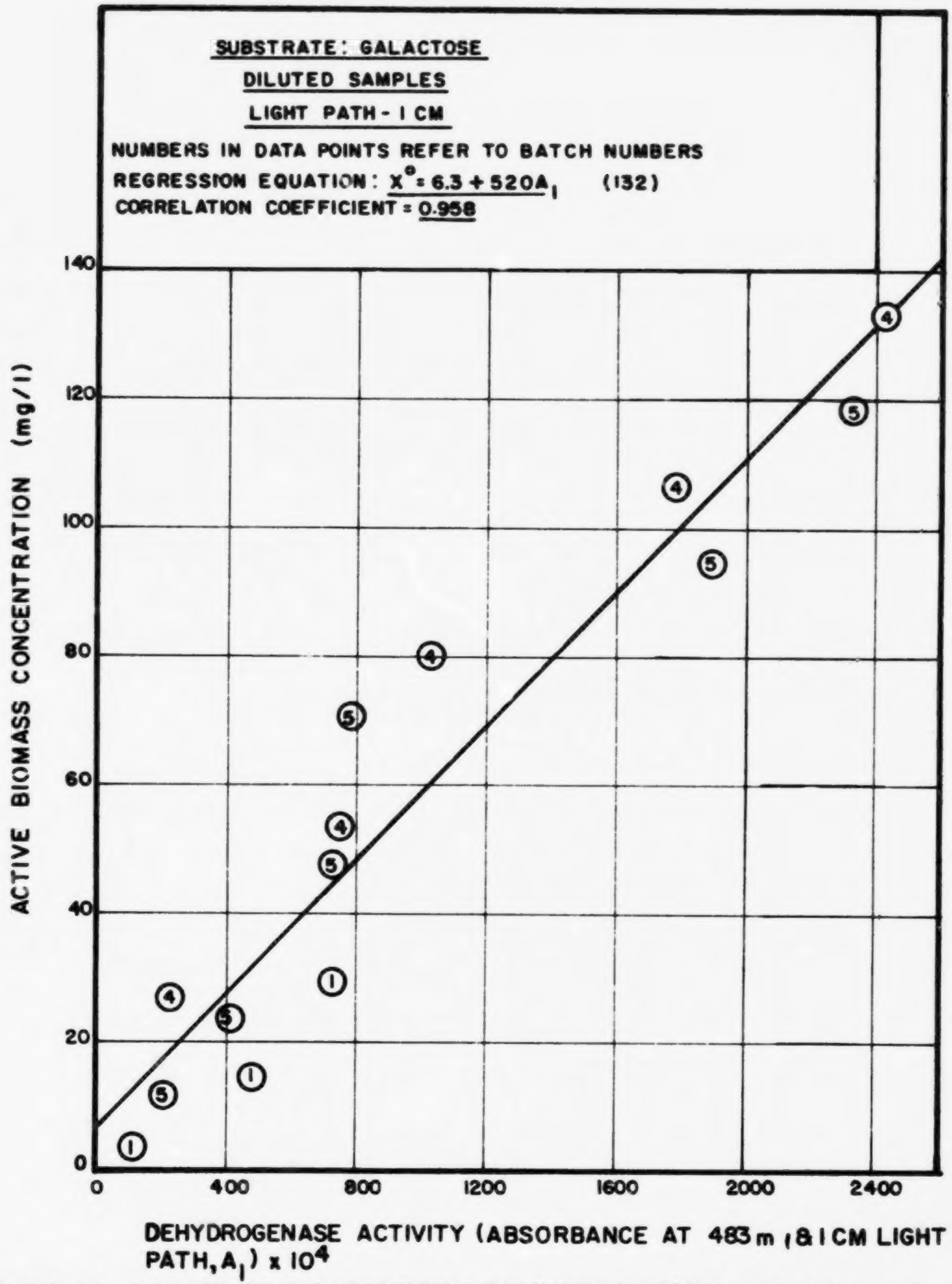


FIGURE 28. CORRELATION BETWEEN DEHYDROGENASE ACTIVITY AND CONCENTRATION OF ACTIVE BIOMASS IN DILUTED SAMPLES OF GALACTOSE GROWN CULTURES.

it is valid to establish this linear relationship from analysis of a single sample at any growth rate after serial dilution.

Figure 29 compares the three regression lines of Figures 26, 27, and 28. Statistical tests of identity of the three lines indicated no significant difference between the three regression lines at the five percent level of significance. The essence of the result of the statistical test was that glucose and galactose grown cultures obeyed the same relationship between dehydrogenase activity (as measured with one centimeter light path) and biomass concentration at all specific growth rates. The solid regression Line 4 of Figure 29 correlates all data from straight and diluted samples of all batch cultures fed with glucose or galactose. The fact that the 95 percent confidence limits of Line 4 enclosed all of the three different regression lines of Equations 130, 131, and 132 provided further evidence that there was little difference between Lines 1, 2, and 3, all of which were estimates of Line 4. The following relationship, which is the regression equation for Line 4 of Figure 29, may be used for determination of active biomass concentration from dehydrogenase activity measured with a one centimeter light path, irrespective of the growth rate of the culture, and regardless of whether the substrate was glucose or galactose:

$$X^{o} = 4.4 + 536\ A_1 \qquad (135)$$

The significance of the intercept of Equation 135 is that solids concentration lower than 4.4 mg/ℓ could not be detected when a light path of one cm was used.

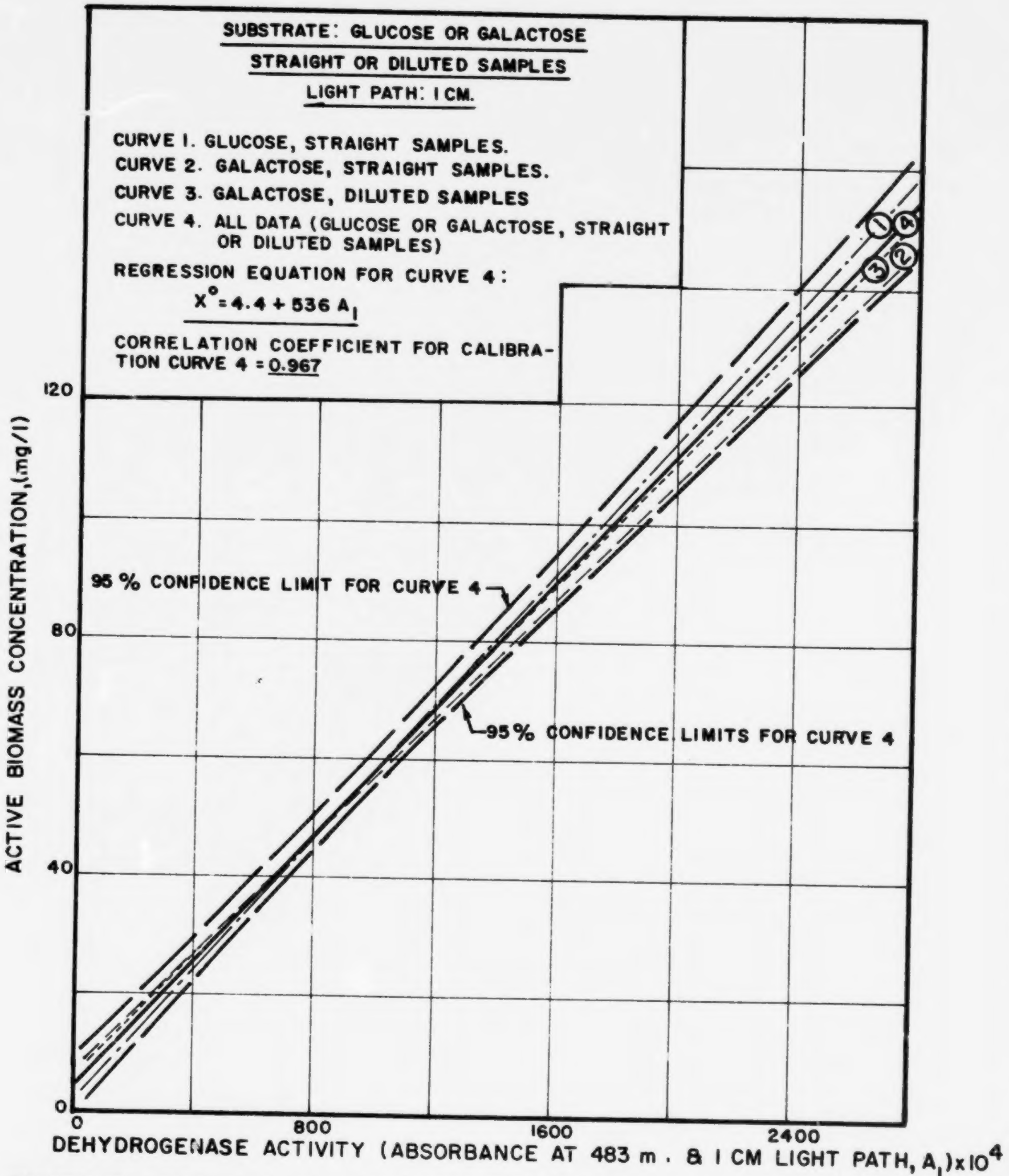


FIGURE 29. CORRELATION BETWEEN DEHYDROGENASE ACTIVITY AND CONCENTRATION OF ACTIVE BIOMASS IN BATCH CULTURES GROWN ON GLUCOSE OR GALACTOSE.

According to the findings of Maxwell (300) and Wilson and Hogness (301,302), galactose metabolism involves the use of an additional NAD serving as a tightly bound prosthetic group of the UDP--galactose-epimerase (galacto-waldenase) not required in the metabolism of glucose (see Figure 4). The mechanism so postulated indicates that the NAD of the epimerase is reduced and then oxidized during a Walden-type conversion of UDP-glucose to UDP-galactose (or vice versa) and, unlike NAD of other dehydrogenases, does not need a separate step for oxidation. Whether TTC can react with the reduced NAD of the epimerase in competition with the usual process of oxidation of this NAD is a moot question. In the event that TTC is successful in oxidizing this epimerase-$NADH_2$, there would be increased production of TF in galactose-grown cultures compared to the TF production for the same concentration of glucose-grown cells. Figure 29 shows that, for biomass concentrations above 50 mg/ℓ, the dehydrogenase activities of galactose-grown cultures (Curve 2) were slightly higher than those for glucose-grown cells (Curve 1) if compared for the same solids concentrations. However, such observed differences were not very significant statistically.

For very dilute cell suspensions, reliable absorbance readings could not be obtained with a one centimeter light path and a longer light path of 10 centimeters was used. A calibration curve for a 10 centimeter light path, based on glucose or galactose grown cultures and for straight or diluted samples, is shown in Figure 30 along with the 95 percent confidence limits of the curve. The relationship for dehydrogenase activity with a 10 centimeter light path and active biomass concentration is given by the following regression equation:

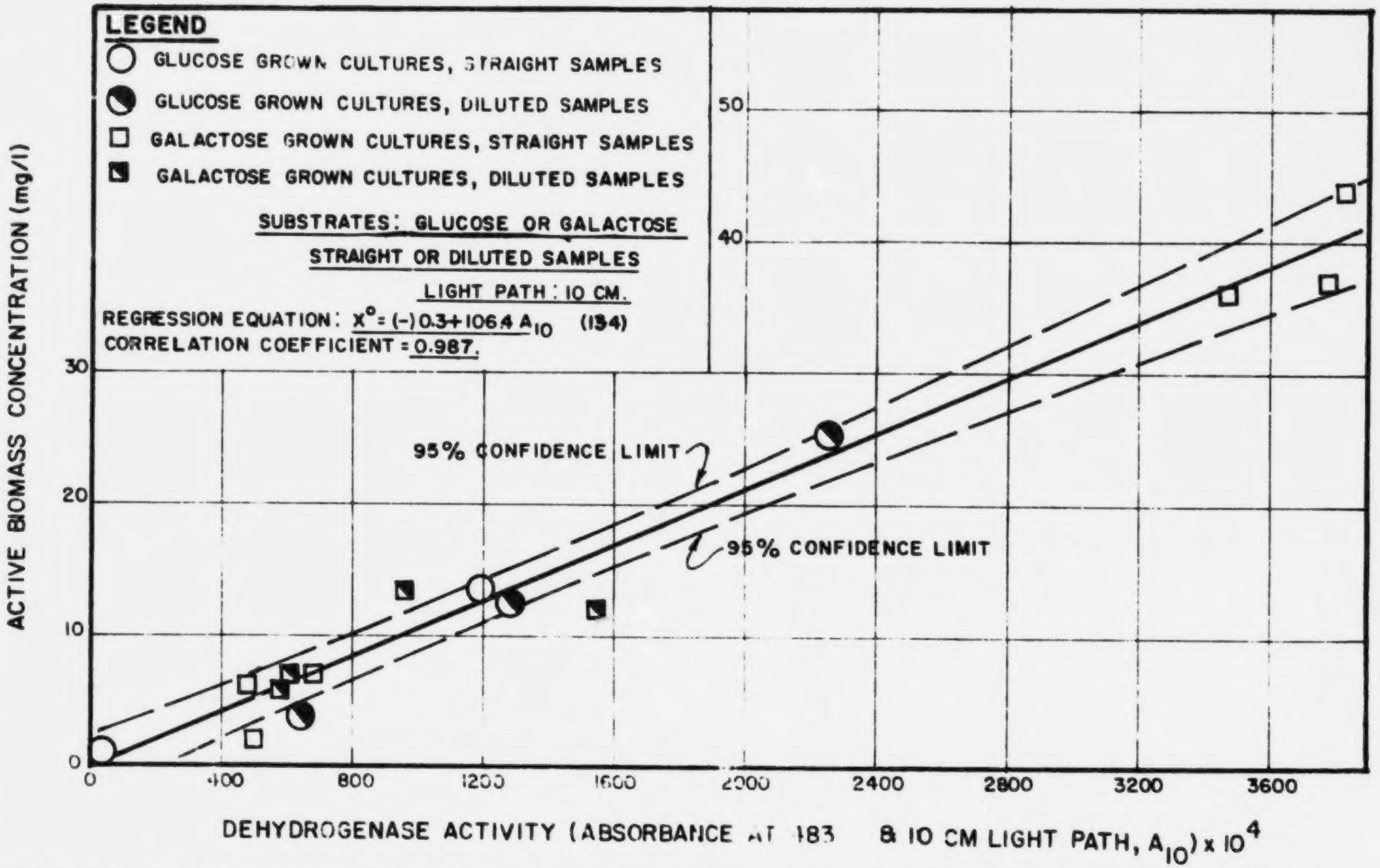


FIGURE 30. CORRELATION BETWEEN DEHYDROGENASE ACTIVITY AND CONCENTRATION OF ACTIVE BIOMASS IN VERY DILUTE BATCH CULTURES GROWN ON GLUCOSE OR GALACTOSE.

$$X^{o} = 106.4\ A_{10} \qquad (136)$$

where $A_{10}$ = absorbance with 10 cm light path.

Figures 26 through 30 have established that the absorbance of tetraformozan is proportional to concentrations of biomass for a given depth of solution and indicate adherence of the TF solution to Beer's law in the range of concentrations tested and above about five mg/ℓ of solids. However, Figure 31 shows that the test solutions did not obey Lambert's (Bouguer's) law[*] since the absorbances of samples with a 10 cm light path were about seven times (instead of 10 times) the values obtained by analysis with a one cm light path. The data of Figure 31, obtained by analyzing samples from cultures under various environmental conditions, are given in Appendix VII. The reasons for the non-adherence of the dehydrogenase solution to Lambert's law are not clear, but whatever they might be, the observations point to the necessity of having separate calibration curves when different light paths are used. Figure 31 indicated that a zero absorbance reading with a one cm cell would be read as an absorbance of 0.0547 if a 10 cm cell were used. A biomass concentration of 5.1 mg/ℓ (from Figure 30) will therefore go undetected with a one cm light path. The regression equations of Figure 29 also indicated that an average biomass concentration of 4.4 mg/ℓ would not cause any absorbance if measured in a one cm cell.

---

[*] Lambert's law:

$$A = \frac{C}{2.303}\,\ell \qquad (137)$$

where A = absorbance by sample having a light path, ℓ
C = a proportionality constant

From Equation 137 the ratio of absorbances in 10 and 1 cm light paths is given by

$$A_{10}/A_{1} = 10 \qquad (138)$$

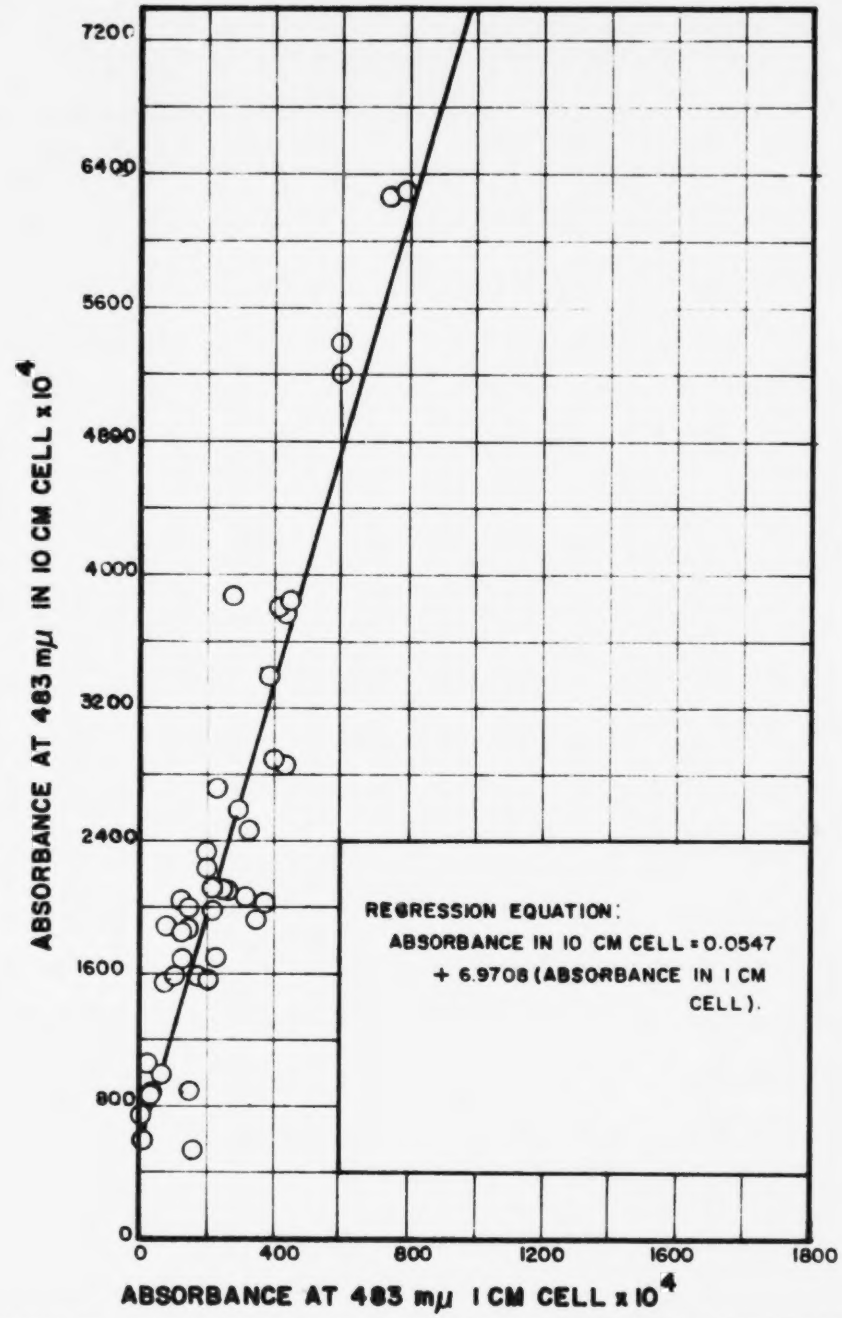


FIGURE 31. RELATIONSHIP BETWEEN OBSERVED ABSORBANCES OF TRIPHENYLFORMOZAN WITH ONE AND TEN CENTIMETER LIGHT PATHS. VERIFICATION OF LAMBERT'S LAW.

The following relationships were used for determination of active biomass concentrations in continuous cultures; the equations were applicable at all specific growth rates as described earlier.

For cultures grown on glucose,

$$X^o = 1.9 + 560\ A_1 \qquad (132)$$

For cultures grown on galactose,

$$X^o = 6.8 + 498\ A_1 \qquad (133)$$

For cultures grown on glucose-galactose mixtures,

$$X^o = 4.4 + 563\ A_1 \qquad (135)$$

For very dilute cultures grown on glucose or galactose,

$$X^o = 106.4\ A_{10} \qquad (136)$$

One unit of absorbance in a one centimeter cell was equivalent to about 540 mg/ℓ of active biomass. On the other hand, one unit of absorbance with a 10 centimeter light path was equivalent to 106 mg/ℓ of active biomass which was in good agreement with Marlar's figure of 100 mg/ℓ per unit of absorbance in 10 cm cells for cultures grown on glucose at 20°C (221).

It may be concluded from the above discussions that, for a given substrate-organism system, the slope of plots of active biomass concentration versus enzyme activity was constant at all growth rates; this meant that the ratio of amount of enzyme synthesized/total new growth is constant. In a given culture the dehydrogenases which satisfy the following conditions are oxidized by triphenyltetrazolium chloride:

Hall-cell potential for $E\text{-}NADH_2 \rightarrow E\text{-}NAD >$ Half-cell potential for $TF \leftarrow TTC$

The extent of reduction of TTC, i.e., the intensity of color formation per unit quantity of dehydrogenase, is dependent on the temperature and duration of reaction, pH, accessibility of the dehydrogenases to TTC, degree of interference by molecular oxygen, any growth of the organism during the reaction, etc. For the procedure used in this research, the absorbances of colored triphenyltetraformozan did not follow the Lambert law.

## Analytical Techniques for Determination of Glucose and Galactose Concentrations

### Determination of Glucose

The reliability of the Somogyi titrimetric method (372) of glucose determination in biological materials has been well established (373, 374). Photometric adaptation of the Somogyi method is also available (375). However, for reasons of simplicity and specificity towards glucose, the enzymatic test involving the catalytic oxidation of glucose to gluconic acid in the presence of glucose oxidase (see Equation 139) (376-378) was preferred. Keston (379) developed a colorimetric test by coupling the above reaction with a peroxidase catalyzed step in which the hydrogen peroxide produced in the presence of oxidase would cause o-tolidine color change due to an oxidation reaction promoted by the peroxidase. The enzymatic reactions are:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2 \qquad (139)$$

$$H_2O_2 + \underset{\text{(Chromogen)}}{\text{o-tolidine}} \xrightarrow{\text{Peroxidase}} H_2O + \tfrac{1}{2}O_2 + \text{Colored Compound} \qquad (140)$$

The intensity of the color of the oxidized chromogenic product is proportional to the amount of glucose present.

Huyggett and Nixon (380,381) developed a procedure using horseradish peroxidase and o-dianisidine as chromogen in lieu of o-tolidine, and Hill and Kessler (382), Discombe (383), and Robin and Saifer (384) adapted this procedure to the autoanalyzer. Jeris and Cardenas (385) have also applied the autoanalyzer technique to wastewater treatment processes.

A more recent technique for continuous analysis of glucose, using the oxidase reaction and residual oxygen concentrations only as indications of glucose concentration, has been reported (386).

In this research, the Worthington Glucostat procedure (387) similar to the procedure of Keston (379) was used. A disadvantage of the procedure was that even for the same glucose concentration different color intensities were obtained when analyzed after slightly different incubation times. A standard calibration curve could not be easily established. The problem was circumvented by running a standard glucose solution of suitable known concentration each time a set of samples was analyzed.

Some modifications of the Worthington procedure were necessary. Since glucose concentrations of the order of one mg/ℓ were to be measured, more reagents and longer incubation periods were used. The samples were filtered to rid them of bacterial solids which would otherwise assimilate glucose during the test period. Millipore filtration was not suitable because the cellulose content of the filters caused three to fourteen percent reduction (depending on the sugar concentration in the sample, filter weight, and rate of filtration) in the sample glucose concentration. Four layers of glass fiber filters were used, although in extremely dispersed suspensions a very clear filtrate could not be obtained from time to time. Any error due to absorbance by turbidity in the glucostat solutions was corrected by measuring the absorbance of a suitably diluted sample and applying the same as correction to the absorbance of the oxidized chromogen. A detailed procedure for the Glucostat test has been included in Appendix VIII.

## Determination of Galactose

For the same reasons underlying the selection of the Glucostat test, an enzymatic test for galactose (the Worthington Galactostat system (388)) was used. The procedure recommended by Worthington did not produce color intensities adequate for reliable measurements for galactose concentrations below 50 mg/$\ell$. Accordingly, a modified procedure, as given in Appendix IX, with increased incubation period, increased quantity of reagent, provision for filtration of samples, and correction for turbidity was used.

# CHAPTER VII

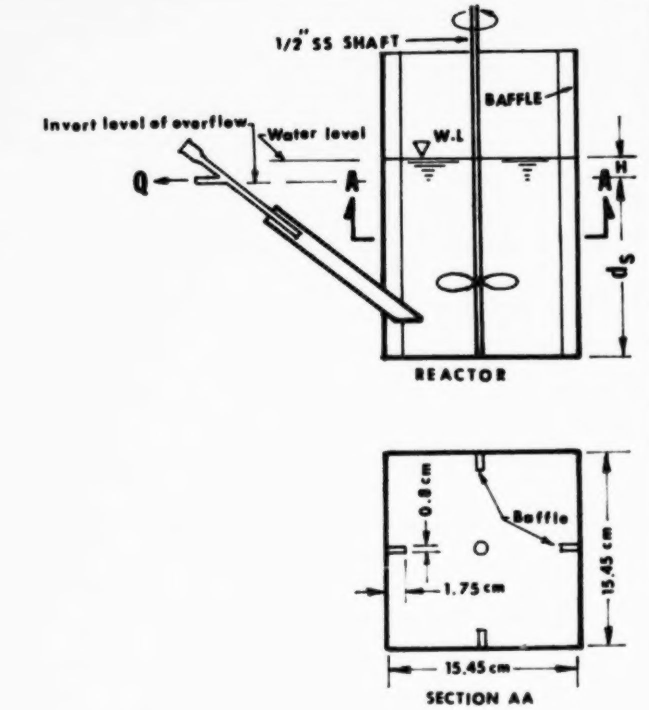
# EXPERIMENTAL PROCEDURE

## Determination of the Hydrodynamic Characteristics of the Reactor Culture Volumes at Various Flow Rates

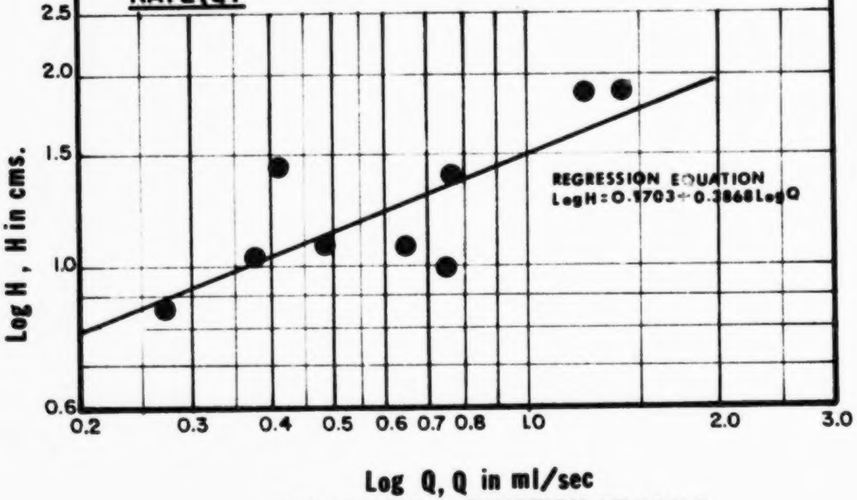
The determinations of reactor detention times or dilution rates, oxygen transfer rates, mixing patterns, and residence-time distributions of reactants in the reactor are all based on the volume of reactor fluids under conditions of turbulence and continuous flow. From Figure 32 it is clear that the volume of the culture depends on the static depth, $d_s$, and the head, H, relative to the invert level of the overflow tube. Despite the constant level of the overflow tube and fixed degree of turbulence in the reactor, the volume of the culture varies with flow rate as $\underline{H}$ varies likewise. Due to the undulating nature of the fluid surface and the small magnitude of $\underline{H}$ and its variation from one flow rate to another, the total depth of reactor fluids $(H+d_s)$ or its variation with flow rate could not be determined accurately by direct measurement during operation of the reactor. It was, therefore, decided to establish a hydraulic relationship between the culture volume and the flow rate so that the former could be determined from measurement of the latter.

The discharge through the orifice outlet is given by an equation of the form

$$Q = C_d A_o \, (2gH)^{1/n} \tag{141}$$


1/2" SS SHAFT
BAFFLE
Invert level of overflow
Water level
W.L
Q
A
H
$d_s$
REACTOR
0.8 cm
Baffle
1.75 cm
15.45 cm
15.45 cm
SECTION AA

A. SCHEMATIC DIAGRAM SHOWING HEAD, H, FOR FLOW RATE, Q.



B. LOGH AS A FUNCTION OF LOG Q

FIGURE 32. VARIATION OF CULTURE VOLUME WITH FLOW RATE IN CONTINUOUS FLOW REACTOR.

where $Q$ = flow rate of effluent = flow rate of substrate + flow rate of nutrients

$A_o$ = cross sectional area of the orifice

$n$ = constant

$C_d$ = coefficient of discharge through the orifice

$H$ = height of water level in reactor above the invert of orifice.

Rearranging Equation 141

$$H = \frac{Q^n}{2g(C_dA_o)^n} \tag{142}$$

Taking common logarithm of both sides of Equation 142

$$\log H = \log\left[\frac{1}{2g(C_dA_o)^n}\right] + n\log Q \tag{143}$$

Also, it is evident from Figure 32 that

$$V = V_s + A_rH \tag{144}$$

where $V_s = A_rD_s$ = constant

$d_s$ = depth of reactor up to invert of overflow = constant (see Figure 32)

$V$ = total volume of reactor

$A_r$ = cross sectional area of the reactor.

Rearranging Equation 144

$$H = \frac{(V-V_s)}{A_r} \tag{145}$$

With the constant level overflow fixed in one position, continuous measurements of V and Q were made with tap water at various flow rates. The value of H for each flow rate was determined from the measured volume at that flow rate from Equation 145 as shown in Table 6. The constants of Equation 143 could then be determined from regression analysis of log H and log Q.

Table 6. Reactor Volume as a Function of Flow-Through Rate

| Measured Flow Rate, Q |  | Measured Volume, V | $V_s$ | $A_r$ | $(V-V_s)$ | H(from Eq. 145) | log H | log Q |
|---|---|---|---|---|---|---|---|---|
| (l/hr) | (ml/sec) | (ml) | (ml) | $(cm^2)$ | (ml) | (cm) |  |  |
| 0.975 | 0.271 | 5670 | 5471 | 231.63 | 199 | 0.859 | -0.0660 | -0.5670 |
| 1.350 | 0.375 | 5710 |  |  | 239 | 1.034 | 0.0145 | -0.4260 |
| 1.480 | 0.411 | 5800 |  |  | 329 | 1.420 | 0.1523 | -0.3862 |
| 1.750 | 0.485 | 5720 |  |  | 249 | 1.075 | 0.0315 | -0.3143 |
| 2.310 | 0.641 | 5715 |  |  | 244 | 1.055 | 0.0233 | -0.1931 |
| 2.710 | 0.752 | 5700 |  |  | 229 | 0.989 | -0.0048 | -0.1238 |
| 2.740 | 0.760 | 5790 |  |  | 319 | 1.379 | 0.1396 | -0.1192 |
| 4.420 | 1.227 | 5900 |  |  | 429 | 1.854 | 0.2681 | 0.0888 |
| 5.090 | 1.410 | 5900 |  |  | 429 | 1.854 | 0.2681 | 0.1492 |

Figure 32 shows a plot of log H vs. log Q and the regression equation describing H as a function of Q. Thus, culture volume at any flow rate was determined from Equation 146 and Equation 144.

$$\log H = 0.1703 + 0.3868 \log Q \qquad (146)$$

Oxygen Transfer Rate

It was necessary for the mechanical and diffused aeration system to be capable of transferring oxygen at a rate higher than the respiration rate of the culture. The respiration rate of the culture, on the other hand, depends on the growth rate of organisms and mass concentration of the same, which in turn is dependent on the substrate consumption at the particular growth rate. The overall oxygen transfer coefficient, $K_La$, required of the aeration system for satisfying the respiration rates was estimated from the following relationship:

$$K_La = \frac{R(X_0^S - X_1^S)Y^o}{\theta_r(\alpha X_s^{O_2} - X_1^{O_2})} \qquad (147)$$

The desired maximum and minimum values of $K_La$ of the oxygen transfer system employed were estimated to be 3.93 $hr^{-1}$ and 0.93 $hr^{-1}$, respectively. The derivation of this relationship and the necessary calculations are presented in Appendix X.

Transfer rate by mechanical agitation could be varied by altering the rotation and peripheral speed of the impellor with the aid of the Zero-Max drive. The reoxygenation capacity of the agitation device at the maximum speed of 400 rpm was determined by separately reaerating deoxygenated tap water and a sterilized culture containing a concentration of microbial solids of 67 mg/ℓ. The curves of oxygen absorption from the atmosphere are shown in Figure 33.

Usually $K_La$ is determined from the slope of a linear plot of $\log(X_s^{O_2} - X_\theta^{O_2})$ vs. $\theta$. However, since the oxygen saturation concentration, $X_s^{O_2}$, is usually an unknown constant and difficult to be determined, a

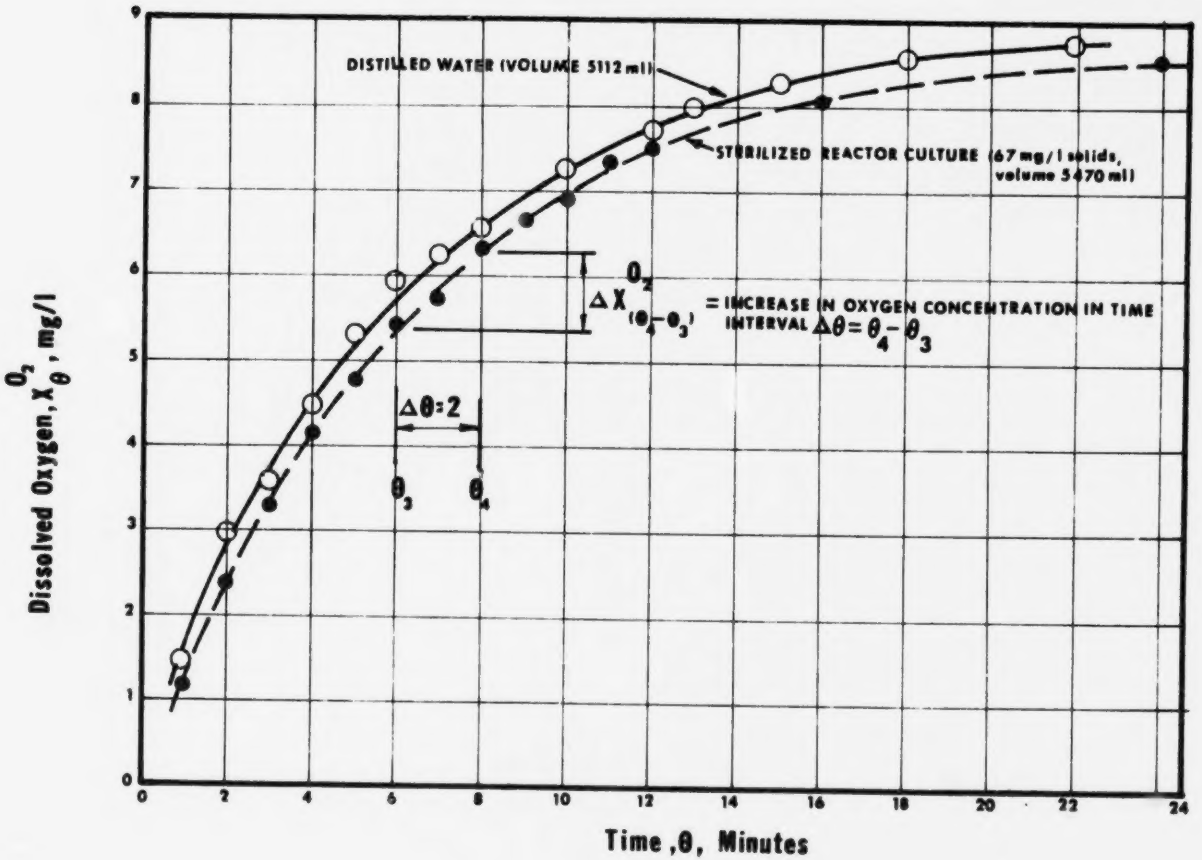


FIGURE 33. RATE OF OXYGEN ABSORPTION BY REACTOR CONTENTS AT 20°C AND MIXING SPEED OF 400 RPM

plot is made with an assumed value of $X_s^{O_2}$ ; if the plot is not linear, then this procedure is repeated until a linear plot is obtained. It may be recognized that this is a very tedious procedure. A graphical technique as illustrated in Figures 33 and 34 was developed as a means of a straightforward and quick determination of $K_La$. Equation 148 (see Appendix XI for derivation) provided the theoretical basis for this technique.

$$\ln \Delta X_{\Delta\theta}^{O_2} = \ln D_0 \left[1-e^{-(K_La)(\Delta\theta)}\right] - K_La\theta \qquad (148)$$

where $\Delta X_{\Delta\theta}^{O_2}$ = incremental oxygen absorption over time interval, $\Delta\theta$

$\Delta\theta$ = a constant time interval

$D_0$ = oxygen deficit at zero time.

From Equation 148 it is evident that the Naperian logarithm of the incremental oxygen absorption over a suitably selected constant interval of time during reoxygenation is a linear function of time; the overall oxygen transfer coefficient is given by the slope of a plot of $\ln \Delta X_{\Delta\theta}^{O_2}$ vs. $\theta$. Such a plot is given in Figure 34 where it is evident that $K_La$ for both tap water and sterilized culture was 10.3 $hr^{-1}$ even though the liquid volumes were slightly different. After reducing the $K_La$ values for the same liquid volume, the coefficient, $\alpha$, was computed to be 1.07 (see Appendix X). It was therefore apparent that mechanical agitation alone at 400 rpm could adequately transfer atmospheric oxygen at a rate higher than the estimated maximum rate of microbial respiration. Furthermore, determination of oxygen transfer capacity by the above procedure appeared to be more convenient than by the method of Cooper, *et al.* (339).

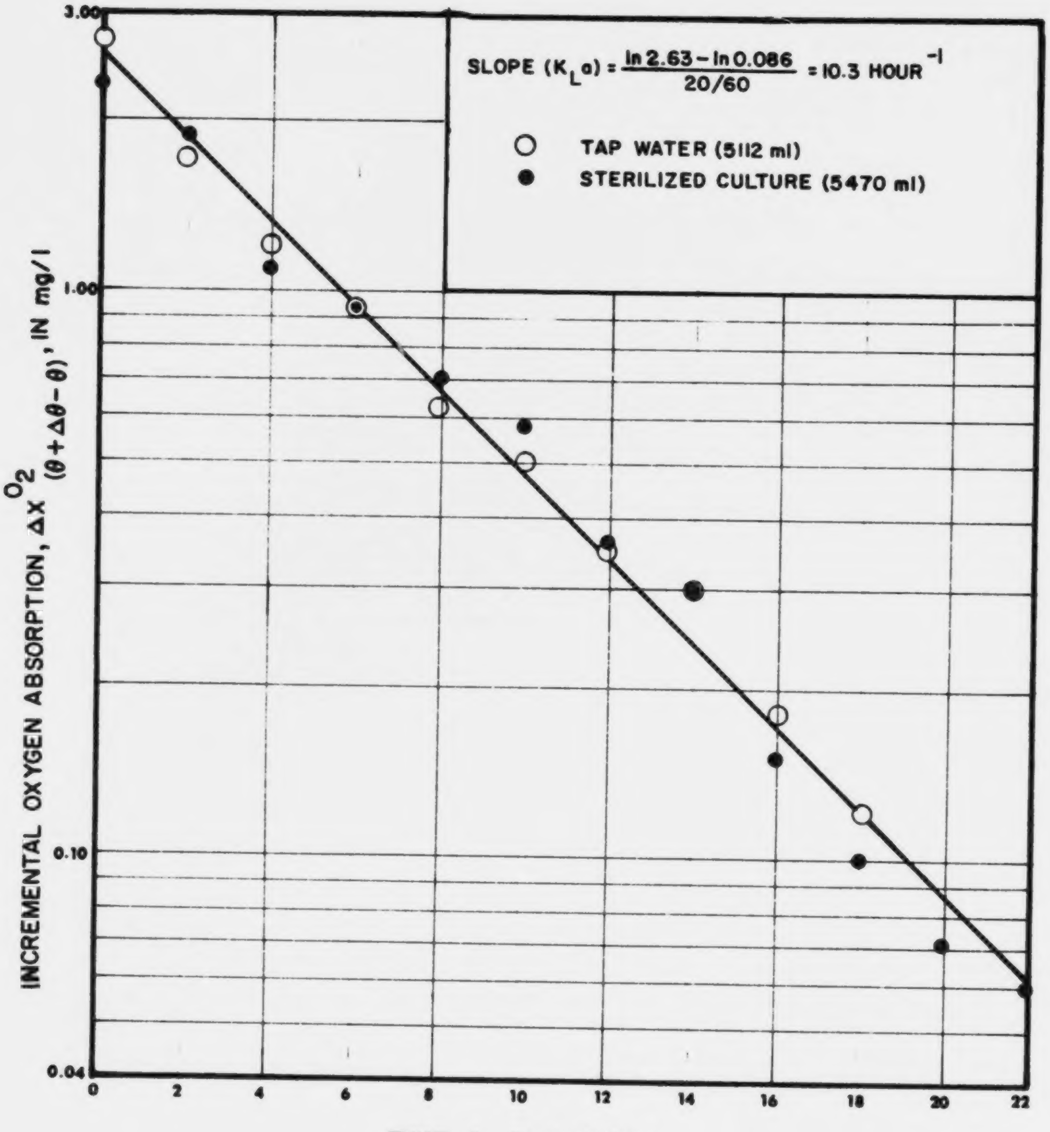


FIGURE 34. DETERMINATION OF OVERALL GAS TRANSFR COFFICIENT, $K_La$, AT 20°C AND MIXING SPEED OF 400 RPM.

## Test for Complete Mix

MacMullin and Weber (390) have shown that the rate of depletion of solute at any time, θ, from a completely-mixed tank initially filled with a solution containing $W^m_o$ mg of solute, due to continuous flow of solute-free solvent will be

$$\frac{dW^m_\theta}{d\theta} = - \frac{\theta}{\theta_r} \qquad (149)$$

where $W^m_\theta$ = weight of solute remaining in tank at time θ and

$\theta_r$ = nominal detention time.

Whence

$$\ln [W^m_\theta / W^m_\theta] = - \theta/\theta_r \qquad (150)$$

or, in terms of concentrations of solute,

$$\ln \frac{X^m_\theta}{X^m_o} = - \frac{\theta}{\theta_r} \qquad (151)$$

Equation 148, which holds only under conditions of complete mixing, could be used to test whether or not complete mixing of the reactor contents was achieved by the agitation system employed. The test reactor was filled with sodium chloride solution at an initial concentration of 3665 mg/ℓ and mixing was started at an impeller speed of 400 rpm. Dilution of the salt solution was next commenced with a continuous flow of distilled and deionized water at a flow rate of 1.15 ℓ/hr. The volume of the salt solution for the chosen flow rate was computed to be 5.7 liters from Equations 144 and 146. The residual salt concentrations at selected times were determined from the values of conductances--measured with a conductivity cell (cell GI, cell constant 1.0 of Industrial Instruments, Inc., Cedar Grove, New Jersey) filled with sample salt solution and forming a resister of a wheatstone bridge (Conductivity bridge, Model RC 16B2, Industrial Instruments, Inc., Cedar Grove, N. J.)--and the calibration curve of Figure 35 established with salt solutions of known concentrations. A plot of $\ln[X_\theta^m/X_o^m]$ as a function of $\theta/\theta_r$ (see Figure 36) yielded a straight line having a slope of -1.02 which compared very well with the theoretical slope of -1.00 predicted from Equation 148 for complete mixing of reactor contents. Mixing at an impeller speed of 400 rpm therefore was considered sufficient to effect complete mixing of the reactor contents.

## Selection and Design of Buffer Solution

As indicated in Chapter V, it was decided to maintain the pH of the continuous culture at the desirable range of 6.7 (325) to 7.0 (328) in order that a heterogeneous system, such as is prevalent in biooxidation processes, could be obtained. Accordingly, a buffer system was designed to neutralize the acidity produced by dissolution of $CO_2$ evolved due to biological activity or by the possible excretion of organic acids (222,235,391) produced at low detention times.

For the desired range of pH, a polyprotic acid buffer system was considered most suitable. Equimolar concentrations of $Na_2HPO_4$ and $KH_2PO_4$ yielded the highest buffer capacity (392) as measured by the buffer index, $\beta$, (393). For a pH change of 0.1 unit due to neutralization of the estimated carbonic acid acidity produced by the culture, the desired buffer

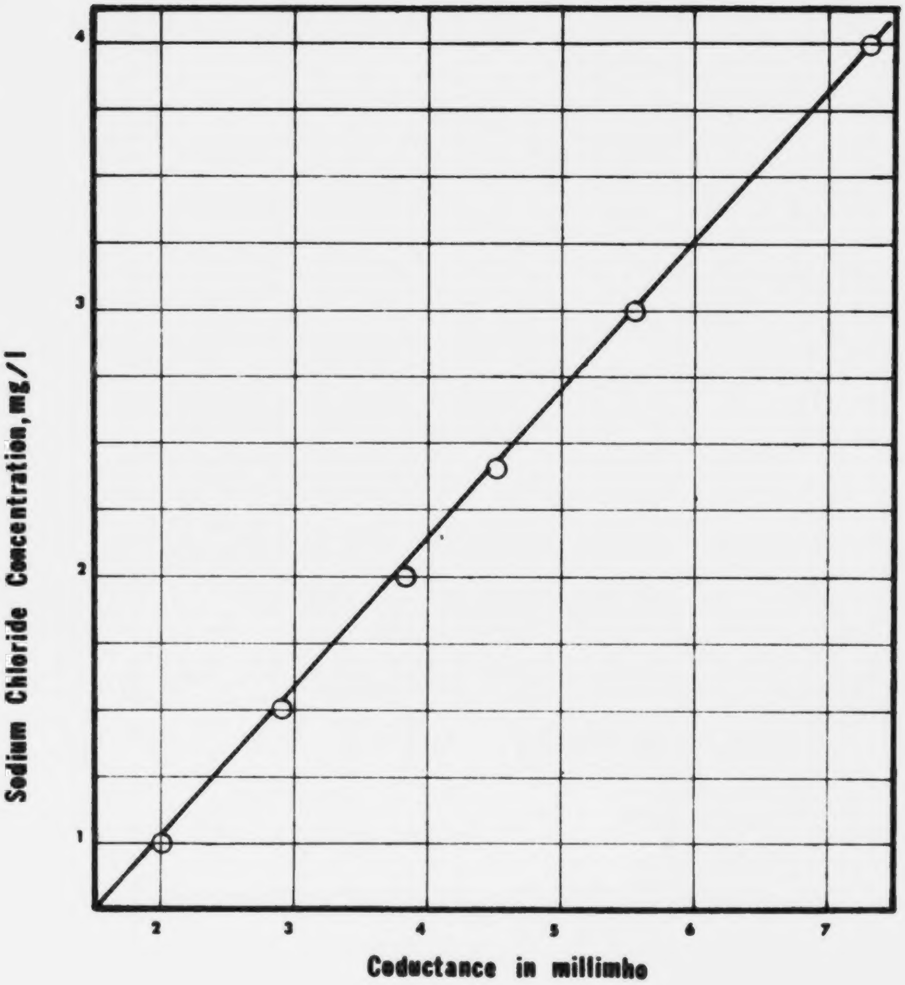


FIGURE 35. CALIBRATION CURVE: CONCENTRATION OF SODIUM CHLORIDE AS A FUNCTION OF CONDUCTANCE

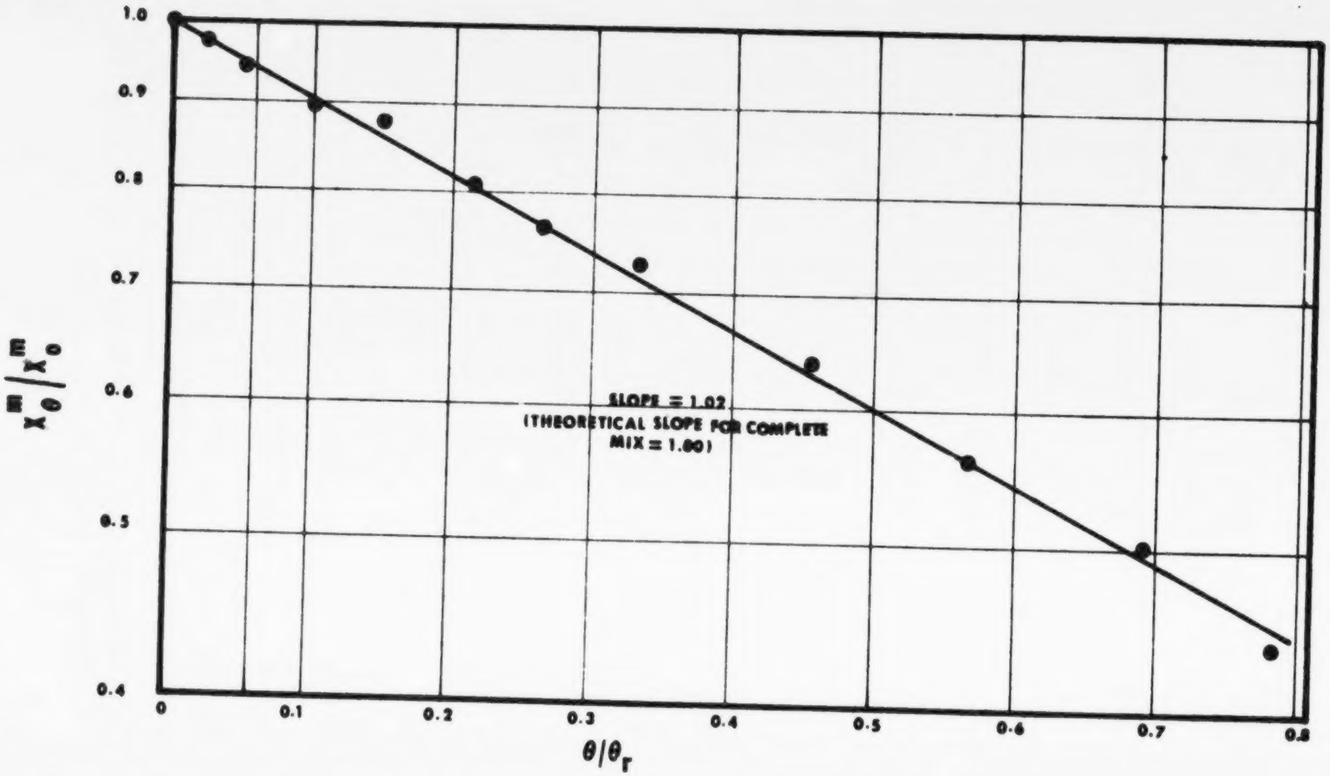


FIGURE 36. EXPONENTIAL DILUTION OF REACTOR SALT SOLUTION MIXED AT IMPELLER SPEED OF 400 RPM

index of the buffer system was calculated to be $4 \times 10^{-2}$ moles/ℓ (in terms of carbonic acid) per unit change in pH (see Appendix XII). Having determined the design buffer index, the concentration of each phosphate of the mixture was computed to be 0.04M. Disodium phosphate ($Na_2HPO_4$) was preferred to dipotassium phosphate ($K_2HPO_4$) since the former chemical was cheaper in bulk quantities and was readily available. As shown in the titration curve of Figure 37, the observed buffer index of the designed buffer was $3.3 \times 10^{-2}$ moles/ℓ in terms of carbonic acid.

## Selection of Influent Concentrations of Substrate Solutions

### General Considerations

The influent concentrations of the substrates have an upper and a lower limit. Denser cultures, resulting from higher concentrations of influent substrate, demand higher oxygen supply and higher oxygen transter rates, cause increased production of $CO_2$, heat and foam, which make pH, temperature, and foam control more difficult. Use of higher influent concentrations means higher costs of chemicals to be used for substrate and buffer solutions. In addition, higher power consumption results for oxygen transfer at enhanced rates and for controlling increased levels of temperature excursion. Measurement of substrate concentration becomes tedious, time consuming, and less accurate as tests have to be performed on diluted samples.

On the other hand, unduly low influent substrate concentrations are undesirable, since the resulting low culture densities lead to inaccuracy in measurement of solids concentrations. In order to improve

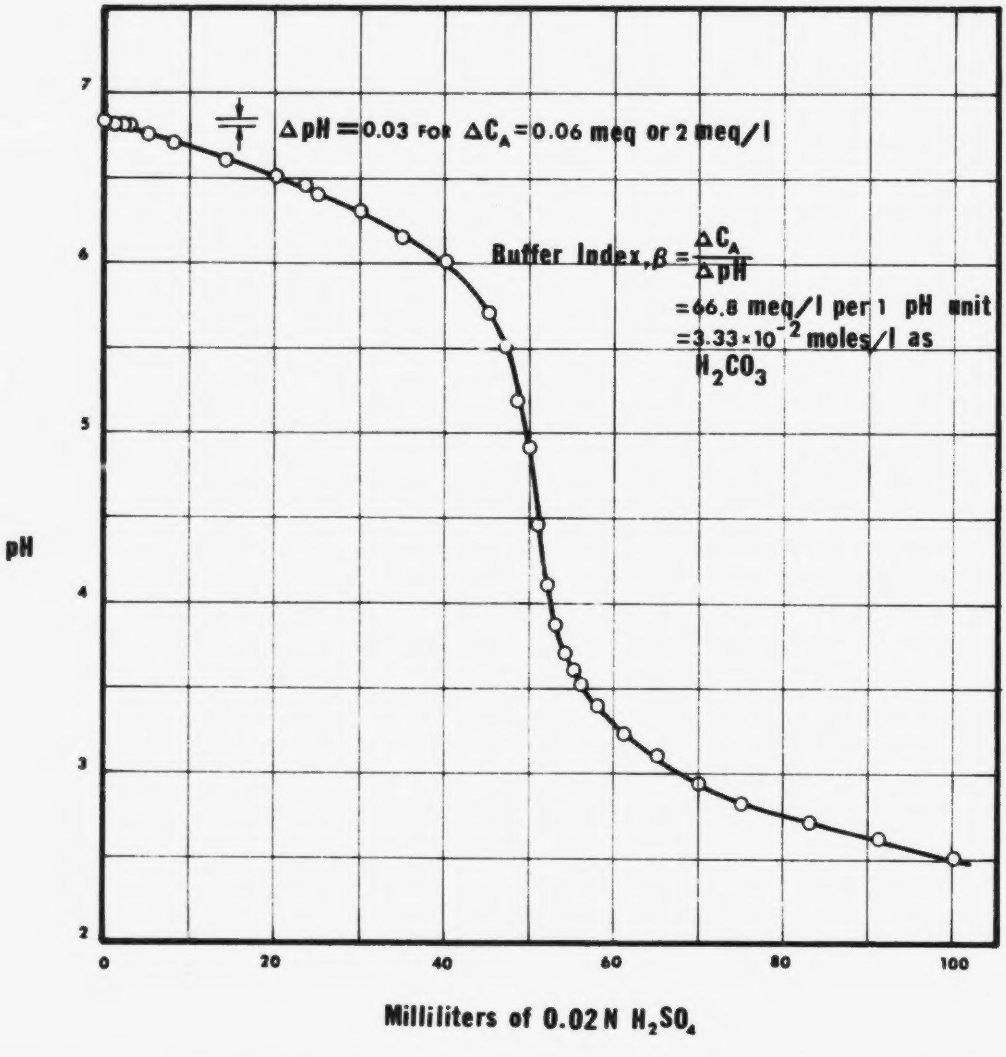


FIGURE 37. TITRATION CURVE FOR DETERMINATION OF BUFFER INDEX OF THE BUFFER SOLUTION USED FOR pH CONTROL.

the accuracy of solids concentration data, sample volume should be large, which is undesirable as it would significantly affect the oxygen transfer rate and the reactor detention time. For each detention time there is a theoretical steady state substrate concentration as given by Equation 64A of Chapter IV. If the selected influent substrate concentration is lower than the theoretical steady state concentration, then no growth occurs.

The selected influent concentrations of substrates were based on a balance of the difficulties anticipated at very high and low substrate concentrations. The selected concentrations were higher than the estimated maximum concentrations in the effluents at the selected detention times. The influent glucose and galactose concentrations ranged from 77 mg/ℓ and 255 mg/ℓ, respectively, and were below the concentration assumed in estimating $K_La$ and buffer concentration. The influent substrate solution was prepared in batches of 44 liters in the 50 liter glass carboy shown in Figures 13 and 17 (of Chapter V) using demineralized water and appropriate volumes of concentrated stock solutions--0.5 g/ℓ for glucose and 0.25 g/ℓ for galactose. The batch solution was mixed by bubbling diffused air for five minutes and then syphoned into the substrate feed reservoir where further mixing was accomplished by magnetic mixing.

## Selection of the Concentrations of Nutrient Solutions

The nutrient or salt solutions were mixed and fed separately in order to provide sources of nitrogen, phosphorus, sodium, potassium, magnesium, iron, calcium, and sulfur. Concentrations used were in excess of the cellular requirements so that growth would be limited only by the concentration of substrate (carbon and energy source) at all growth rates. Estimates of requirements of the elements were based on the recommended C:N ratio of 6:1 (Hoover and Porges (394)), C:P ratio of 20:1 (Eckenfelder and O'Connor (328)), and $P:K^+:Mg^+$ ratios of 8:4:1 (Tempest, et al. (326)). The salt concentrations in the nutrient feed reservoirs were higher than the concentrations computed from the above ratios of elements by a factor representing the extent of dilution of the nutrient concentration by the substrate flows. The concentrations of the various nutrient salts could be computed from the following formula:

$$X_f^{ns} = \left[\frac{M^{ns}}{W^{ne}}\right]\left[X_o^s \cdot \frac{W^c}{M^s} \cdot \frac{1}{R}\right]\left[\frac{Q^t}{Q^n}\right] \qquad (152)$$

where $X_f^{ns}$ = concentration of a nutrient salt in the feed reservoir

$M^{ns}$ = molecular weight of salt serving as a source of an element

$W^{ne}$ = weight of the nutrient element in one formula weight of the salt source

$X_o^s$ = influent substrate concentration after dilution

$W^c$ = weight of carbon in one formula weight of substrate

$M^s$ = molecular weight of substrate

$R$ = parts of carbon for each part of the element

$Q^t$ = total flow rate

$Q^n$ = flow rate of mixed nutrients solution

A sample calculation for the concentration of the nutrient salt furnishing the nitrogen requirement is presented in Appendix XIII. The concentrations of all of the other nutrient salts in the feed reservoir are also presented in Appendix XIII.

The salt solutions were prepared by transferring appropriate quantities of stock solutions of the salts into the nutrient feed reservoir, diluting with deionized water up to 62 liters, and mixing with the motor driven paddles in the reservoirs.

## Selection of Flow Rates

For a desired culture volume--usually between 5.5 to 6.0 liters--a total flow rate was chosen to provide the desired detention time. The substrate and nutrient flow rates were so proportioned that the dilution rate, $Q^t/Q^n$, never exceeded the design ratio of 12 used in the computations of Appendix XIII. Since the actual culture volume was a function of the flow rate, final adjustments of the flow rates to yield the desired detention time were made after continuous flow was started and the culture volume determined for the selected flow-through rate.

## Continuous Runs

Before beginning the continuous flow, a batch culture was grown in the reactor filled with medium containing glucose at a concentration of 193.6 mg/ℓ and buffer and nutrient salts added in amounts prescribed in Appendix IV. In order to develop a heterogeneous microbial population, the medium was seeded with settled and filtered (through glass wool) activated sludge obtained from the South River Plant of the Atlanta Water Pollution Control System. The growth medium was agitated at 400 rpm to ensure complete mixing. Culture temperature was maintained at 20° C. The growth of the culture was followed by sampling for glucose concentrations and dehydrogenase activities at different times. DO levels were recorded continuously.

Continuous flow of glucose and the nutrient salt solutions was started as described in the last section of Chapter V before the batch culture reached the end of the exponential growth phase. A high detention time was selected for the first run so that a maximum heterogeneity of the microbial population could be maintained in the first continuous run and selection of a smaller number of species at a subsequently increased dilution rate could be effected from a larger number of species prevalent at the preceding lower dilution rate. Glucose concentrations in the influent and the reactor as well as the dehydrogenase activities of the mixed liquor were determined at selected times. A few dry solids determinations were performed for each run of a particular detention time. The key points during steady state operations were: (a) maintenance of a homogeneous distribution of organisms by mixing, (b) accurate regulation of the culture volume, (c) accurate regulation of flow rates, (d) accurate regulation of substrate concentration, and (e) accurate control of temperature. These physical environmental factors were controlled following operational procedures described in Chapter V. Sampling for steady state substrate and organism concentrations for the detention time of the run was continued for a duration equal to or more than five nominal detention times.

After the steady state concentrations were established for a detention time, suitable changes were made in the flow rates of substrate and nutrient salts for operation at a lower detention time (i.e., a higher dilution rate). Large changes in dilution rates were avoided as recommended by Herbert, et al. (240). The series of runs with a substrate was so scheduled that there was a stepwise positive shift in dilution rates from one run to the subsequent one. A reverse schedule was undesirable in the mixed culture system since the heterogeneity of the microbial population could not be increased, compatible with increases in detention time, when shifting from an initial environment of low detention time harboring fewer species. An added advantage of such a scheme of operation was that oscillations in the transient state conditions between the two steady states were kept to a minimum as also noted by Mor and Fiechtor (395). These authors have shown that, with "shifting up" of dilution rates, the final steady states are approached asymptotically on changing the dilution rate in contrast to an initial sigmoidal approach if the dilution rate was "shifted down." In other words, the length of transition period between two steady states would be shorter with positive shifts in dilution rates. Mor and Fiechter (395) have also shown that the length of the transition period decreased with decrease in the detention time after the shift and that this duration was approximately 20 hours when the shift was to a detention time of 5.92 hours. Since the detention times in this research never exceeded six hours, a transition period of not more than $4\theta_r$ was expected. Sampling during this estimated transition period was not done in some cases. A transition time of $4\theta_r$ also compared favorably with the observations of Button and Garver (261) that stable conditions were achievable after five fermentor volumes were allowed to pass through the fermentor following the change in dilution rate.

As the heterogeneity of the culture was expected to be greatly reduced at high dilution rates, the reactor was reseeded with organisms from the activated sludge unit of the South River Sewage Treatment Plant of Atlanta after termination of the glucose runs. The reactor was operated at a detention time of about eight hours for two days before beginning the galactose series in order to achieve a heterogeneity compatible with the environment associated at low rates of hydraulic displacement.

Procedures for sampling and operation of the continuous culture system during the galactose runs were essentially similar to what have been described for the glucose runs.

The last series of runs was made with mixtures of glucose and galactose fed as the carbon and energy sources, starting the series following reseeding and operation at a high detention time as mentioned above. Influent and reactor samples were analyzed for both glucose and galactose while the other steps of the procedure were similar to those of the glucose and the galactose runs.

## Bacteriological Examinations

Delineation of the taxonomic composition of the culture at a given dilution rate and its variation from one dilution rate to another calls for the study of morphological characteristics, staining properties, enrichment and subculturing, isolation of species, nutritional characteristics, fermentative, and other metabolic properties and identification of the different species which were outside the scope of this investigation. Nevertheless, the following simpler examinations were made of the cultures grown at a low and a high detention time for each of the two substrates in order to ascertain if there existed any easily detectable indications of difference in compositions of microbial populations at the two extreme values of the dilution rates used in the series of continuous runs:

1. Microscopic examination for morphological variation.

2. Physical characteristics (shape, size, appearance, odor, etc.) of colonies grown on tryptone-glucose extract agar of Standard Methods (327).

3. Gram staining properties by the Gram stain technique of Standard Methods (327).

4. Presence of coliform groups by the membrane filter technique (327).

5. Pigment formation and light absorption properties of alcohol extractible pigments (Cassell, *et al*. (270)).

## Problems in the Attainment of Steady States

### Nature of the Problem

Adherence of bacteria on solid-liquid interfaces and irregular sloughing of slime growth proved to be a serious menace to the achievement of stable steady states. Due to inconsistencies in data and highly oscillatory concentrations of substrate and organism, a few runs had to be rejected and repeated in the initial phases of the research. An additional search of the literature was undertaken to ascertain if there existed any technique which could be used to alleviate this serious problem.

### Experiences of Other Investigators

Slime growths on reactor surfaces have often been the cause of poor experimental results (62-65,261,225,239,252,253). Holme (234) reported on the termination of many experiments due to severe slime formation by *E. coli B.* Button and Garver (261) used *T. utilis* in lieu of *E. coli* due to the slime forming properties of the latter. Bryson (252) has reported on erroneous functioning of turbidostat due to "wall growth" in the growth tube.

It appears that the extent of wall growth depends on the adhesiveness of a particular strain of bacteria (252,261), the chemical nature of the growth medium (252), and the nature of the solid surfaces (261, 252). Wall growth is most predominant at lower dilution rates and with heterogeneous culture (239,225,226). Dias, *et al*. (396) have reported that, in heterogeneous cultures grown on sewage and glucose, filamentous sphaerotilus was the dominant organism of the slime layer at high dilution rates, at pH values of 7.4 or more, low oxygen levels, and in the presence of nitrogen and phosphorus deficiencies.

Failure to attain stability in the presence of slime is mainly attributed to the creation of a non-homoegneous culture--partly in the solid and partly in the liquid phase--which is contrary to the assumption in the derivation of the steady state equations. Because of adhesion on solid surfaces, growth of the slime cannot be balanced by hydraulic dilution and the basic premise of continuous culture theory that net specific growth rate is equal to the dilution rate does not hold. Slime growth thus led to unsteady situations. When adherent cell population forms a considerable fraction of the total reactor cell population of low density obtained at higher dilution rates, serious deviation of observed density from the theoretical values may be expected. Situations may be encountered where the bacterial concentration in the mixed liquor may be almost zero at high dilution rates exceeding $k^m$, and yet complete washouts in terms of substrate utilization cannot be realized due to growth and assimilation of the substrate by the attached organisms. Due to higher consumption of substrates at lower dilution rates, slime was formed in thicker layers and sloughed off the solid surfaces from time to time, thereby causing momentary increase in organisms concentration accompanied by a decrease in substrate concentration in the reactor. The amplitude of oscillation of substrate concentration relative to the steady state value is expected to be more at lower dilution rates, whereas that of organism concentration is expected to be more at higher dilution rates.

Various remedies such as coating the reactor surface with special compounds (252), vigorous agitation, scouring the reactor surface with glass beads (317) and magnet (320), mechanical scraping of slimes by "windshield wiper" arrangement (252,397), etc. have been used. Unfortunately, none of these methods appeared to be very successful and free from operational difficulties. Some investigators have not used any remedial measures and the reported results are of doubtful significance in cases of slime forming cultures, and when the reactor surface area becomes larger compared to the culture volume. It was apparent that no technique for prevention or control of slime growth was available at the time.

## Technique Used for Controlling Slime Growth

The procedure used for control of slime growth during this research was to thoroughly brush all of the surfaces in contact with the culture with a large test tube brush with heavily filled bristles and attached to a heavy handle of adequate length. The frequency of brushings to dislodge the attached organisms depended on the estimated generation time in a particular run. The interval between two consecutive brushings was not more than the estimated generation time. A very improved degree of stability of the steady states could be attained with this procedure.

# CHAPTER VIII

# PRESENTATION AND DISCUSSION OF THE EXPERIMENTAL DATA

## Presentation of the Experimental Data

The experimental data on continuous culture runs with glucose, galactose, or mixtures of two sugars serving as the growth controlling substrate are presented in tabular form in Appendix XIV. Figures 38 and 39 show the results of typical runs with single substrate and mixed substrate, respectively. All steady states were characterized by oscillations of substrate and organism concentrations about a mean. Slime growth on surfaces exposed to the culture posed a definite impediment to attainment of steady states. Slimes not only caused erratic fluctuations of concentrations of substrate and organisms but also made attainment of steady state impossible when the reactor was operated at dilution rates of 0.3/hr or less.

In the presence of slime growth, the total quantity of organisms in the reactor was more than what would have been present solely as suspended solids at a given dilution rate if only suspended organisms were present. Therefore, as a result of slime, the substrate concentration tended to be much lower (sometimes zero), and the mean organism concentration tended to be much higher than the corresponding steady state concentration predictable from theory. This change of substrate concentration is illustrated in Figure 38. Substrate concentration could be depressed by as much as 50 percent when slime was allowed to accumulate.

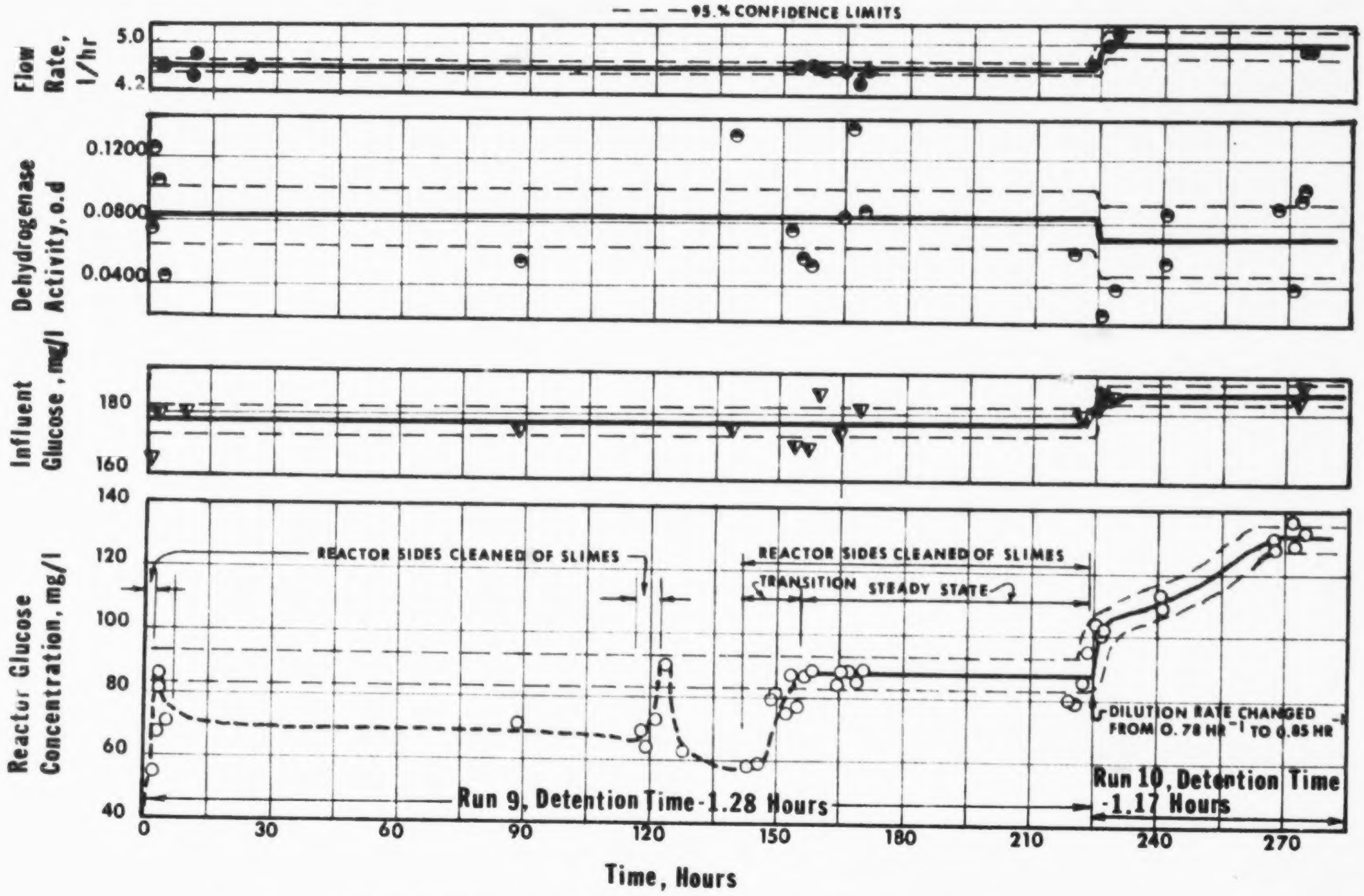


FIGURE 38. TYPICAL STEADY STATE RUN WITH GLUCOSE AS SUBSTRATE

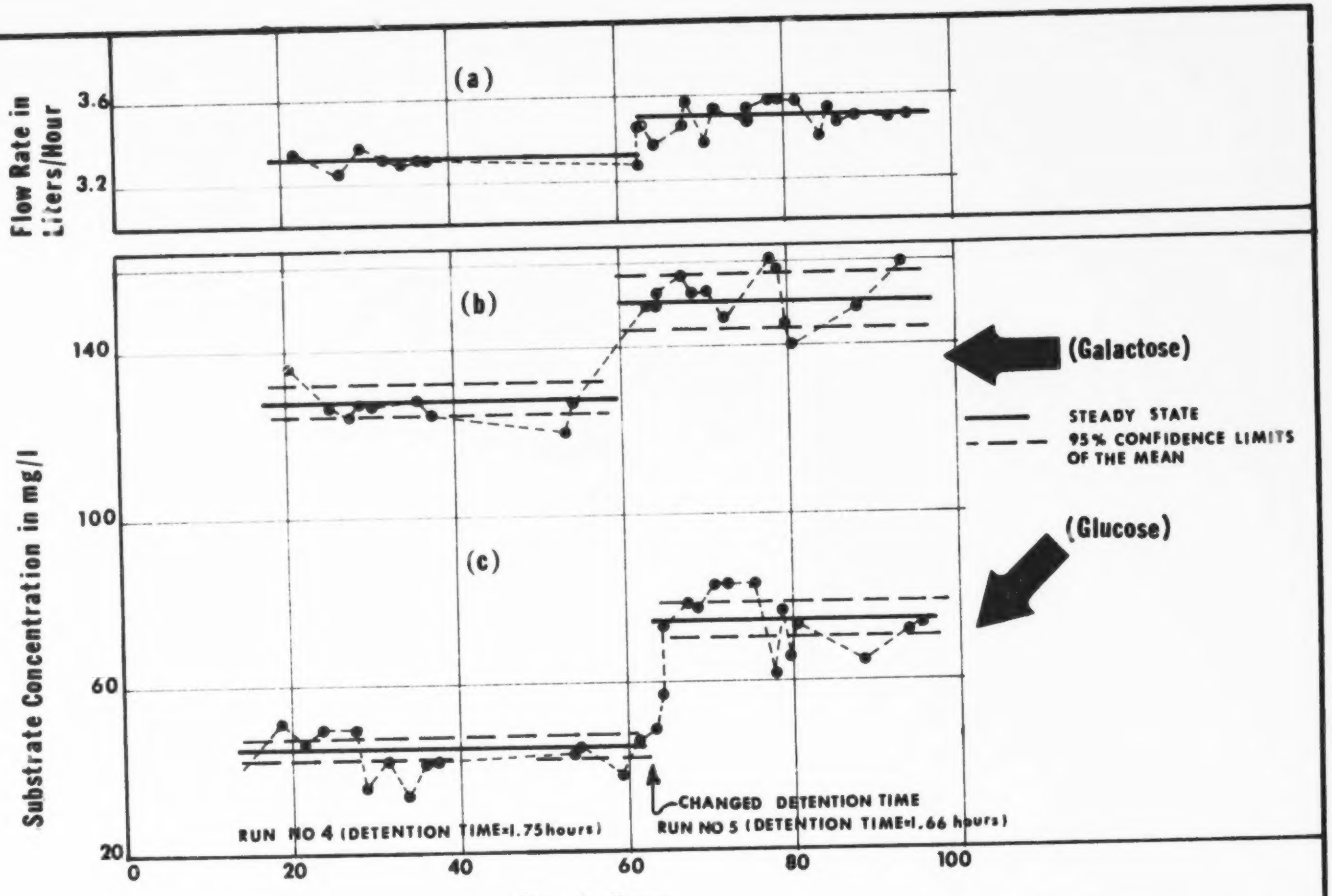


FIGURE 39. A TYPICAL STEADY STATE RUN WITH MIXED SUBSTRATE. FLOW RATE (a), GALACTOSE CONCENTRATION (b) AND GLUCOSE CONCENTRATION (c) AS FUNCTIONS OF TIME.

Some base level fluctuation of steady state concentration was almost inevitable because of the variation in flow rate which could not be avoided completely. Periodic sloughing of slimes gave rise to momentary increases in the organism concentration which were then gradually diluted out with time to the level existing prior to sloughing. Thus, erratic oscillations of solids and substrate concentrations were superimposed on the low base level of fluctuations. Sloughings at low dilution rates were heavy and more frequent since higher levels of solids could be maintained at these dilution rates. Heavy sloughings caused great dips of substrate concentrations, sometimes to the zero level. The plot of Figure 39 is typical of runs in which the reactor was operated without the occurrence of appreciable slime growth.

The mean of the observations, excluding observations taken during transition periods between steady states on any parameter during a run, was taken as the steady state value of the parameter for the detention time of the run. Tables 7, 8, and 9 present the steady state values of parameters for different detention times when glucose, galactose, and mixtures of glucose and galactose were being used as the substrates.

## Discussion of the Experimental Data

### Variability of the Environmental Factors, Substrate, and Organism Concentrations in the Steady State Runs

Because steady state conditions should be characterized by constant values of environmental parameters and substrate and organism concentrations, it was decided to examine the variability of these parameters during a steady state run, as well as the effect of the detention time on the variability of steady states.

Table 7. Summary of Steady State Runs with Glucose as the Substrate

| Run No. | Glucose Conc. | | Dehydrogenase Activity | | Active Organism Conc. | Total Solids Conc. | Flow Rate | Culture Volume | Detention Time | DO | Temp. | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Influent | Reactor | 1 cm Cell | 10 cm Cell | | | | | | | | |
| | $X_o^g$ | $X_1^g$ | $A_1$ | $A_{10}$ | $X_1^o$ | $X_1^{ot}$ | Q | V | $\theta_r$ | | | |
| | (mg/ℓ) | (mg/ℓ) | (o.d.) | (o.d.) | (mg/ℓ) | (mg/ℓ) | (ℓ/hr) | (ℓ) | (hr) | (mg/ℓ) | (°C) | |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 1 | 184.7 | 2.8 | -- | 0.5845 | 62.2 | 91.0 | 1.41 | 5.710 | 4.050 | 6.2 | 20.4 | 6.6 |
| 2 | 135.1 | 3.1 | -- | 0.4568 | 48.6 | 75.3 | 1.84 | 5.735 | 3.117 | 4.6 | 20.4 | 6.8 |
| 3 | 150.4 | 2.5 | -- | 0.4490 | 47.8 | 84.4 | 2.55 | 5.771 | 2.263 | 3.1 | 21.2 | 6.8 |
| 4 | 148.0 | 5.8 | 0.1251 | -- | 72.0 | 85.0 | 2.99 | 5.790 | 1.936 | 6.2 | 20.0 | 6.8 |
| 5 | 77.0 | 5.8 | 0.0734 | -- | 43.0 | 39.1 | 2.99 | 5.790 | 1.936 | 5.6 | 21.0 | 6.8 |
| 6 | 144.3 | 41.1 | 0.0813 | -- | 47.4 | 64.9 | 3.30 | 5.802 | 1.758 | 4.7 | 20.8 | 6.7 |
| 7 | 166.5 | 64.2 | 0.0909 | -- | 52.8 | 65.2 | 3.60 | 5.814 | 1.615 | 7.8 | 19.8 | 6.8 |
| 8 | 222.9 | 85.4 | 0.1196 | -- | 67.8 | 88.7 | 3.84 | 5.823 | 1.516 | 5.2 | 20.5 | 6.7 |
| 9 | 176.8 | 87.6 | 0.0828 | -- | 48.3 | 62.0 | 4.58 | 5.847 | 1.277 | 5.7 | 20.5 | 6.8 |
| 10 | 185.6 | 131.8 | 0.0691 | -- | 40.6 | -- | 5.00 | 5.860 | 1.172 | 9.8 | 20.2 | 6.8 |
| | | | | | | | | | Average | | 20.5 | 6.76 |
| | | | | | | | | | Standard Deviation | | 0.43 | 0.056 |
| | | | | | | | | | Coefficient of Variation (%) | | 2.1 | 0.8 |

Note: Col. 6 computed from Equation 132.

Col. 9 computed from Equations 144 and 146.

Table 8. Summary of Steady State Runs with Galactose as the Substrate

| Run No. | Galactose Conc.<br>Influent<br>$X_o^{ga}$ (mg/ℓ) | Galactose Conc.<br>Reactor<br>$X_1^{ga}$ (mg/ℓ) | Dehydrogenase Activity<br>1 cm Cell<br>$A_1$ (o.d.) | Dehydrogenase Activity<br>10 cm Cell<br>$A_{10}$ (o.d.) | Active Organism Conc.<br>1 cm Cell<br>$X_1^o$ (mg/ℓ) | Active Organism Conc.<br>10 cm Cell<br>$X_1^o$ (mg/ℓ) | Total Solids Conc.<br>$X_1^{ot}$ (mg/ℓ) | Flow Rate<br>Q (ℓ/hr) | Culture Volume<br>V (ℓ) | Detention Time<br>$\theta_r$ (hr) | DO<br>(mg/ℓ) | Temp<br>(°C) | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 1 | 136.4 | 4.3 | 0.1541 | -- | 83.5 | -- | 90.0 | 0.935 | 5.535 | 5.92 | 1.8 | 21.6 | 6.8 |
| 2 | 131.8 | 4.0 | 0.0573 | 0.6010 | 35.3 | 63.9 | 66.0 | 0.950 | 5.539 | 5.83 | 2.0 | 19.8 | 6.8 |
| 3 | 181.2 | 1.9 | 0.1721 | -- | 92.5 | -- | 102.0 | 1.500 | 5.715 | 3.81 | 4.8 | 20.0 | 6.7 |
| 4 | 184.4 | 62.9 | -- | 0.2655 | -- | -- | 72.0 | 1.760 | 5.736 | 3.26 | 6.7 | 19.9 | 6.8 |
| 5 | 185.6 | 87.7 | 0.0666 | -- | 40.0 | -- | 60.0 | 1.940 | 5.741 | 2.96 | 5.3 | 20.0 | 6.8 |
| 6 | 197.4 | 26.8 | 0.1848 | -- | 98.8 | -- | 100.0 | 2.020 | 5.745 | 2.84 | 1.8 | 20.6 | 6.8 |
| 7 | 181.0 | 100.7 | -- | 0.3502 | -- | 37.3 | 40.1 | 2.290 | 5.759 | 2.51 | 6.2 | 20.2 | 6.8 |
| 8 | 187.3 | 116.2 | -- | 0.1068 | -- | 11.4 | 26.7 | 2.740 | 5.780 | 2.11 | 6.7 | 20.1 | 6.8 |
| 9 | 165.2 | 24.0 | 0.0946 | -- | 53.9 | -- | 63.0 | 2.810 | 5.783 | 2.06 | 4.8 | 20.4 | 6.8 |
| 10 | 193.8 | 172.8 | 0.0212 | 0.1344 | 17.4 | 14.3 | 29.4 | 2.990 | 5.790 | 1.94 | 6.0 | 19.5 | 7.1 |
| 11 | 255.8 | 133.9 | 0.0958 | -- | 54.5 | -- | 53.1 | 3.520 | 5.811 | 1.65 | 4.1 | 21.0 | 6.9 |
|  |  |  |  |  |  |  |  |  |  | Average |  | 20.3 | 6.8 |
|  |  |  |  |  |  |  |  |  |  | Standard Deviation |  | 0.6 | 0.11 |
|  |  |  |  |  |  |  |  |  |  | Coefficient of Variation (%) |  | 3.0 | 1.7 |

Note: Figures in columns 7, 8, and 10 are computed from Equations 133, 136, and 144 and 146.

Table 9. Summary of Steady State Runs with Glucose-Galactose Mixtures

| Run No. | Glucose Concentration |  | Galactose Concentration |  | Dehydrogenase Activity |  | Solids Conc. | Flow Rate | Culture Volume* | Detention Time | DO | Temp. | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Influent | Reactor | Influent | Reactor | Light Path |  |  |  |  |  |  |  |  |
|  |  |  |  |  | 1 cm | 10 cm |  |  |  |  |  |  |  |
|  | $X_0^g$ (mg/ℓ) | $X_1^g$ (mg/ℓ) | $X_0^{ga}$ (mg/ℓ) | $X_1^{ga}$ (mg/ℓ) | (o.d.) | (o.d.) | $X_1^{ot}$ (mg/ℓ) | Q (ℓ/hr) | V (ℓ) | $\theta_r$ (hrs) | (mg/ℓ) | (°C) |  |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 1 | 113.9 | 3.2 | 119.9 | 3.1 | 0.1615 | -- | 114.0 | 2.02 | 5.745 | 2.84 | 2.0 | 19.3 | 6.80 |
| 2 | 120.7 | 1.7 | 153.4 | 37.2 | 0.1441 | -- | 110.0 | 2.62 | 5.774 | 2.20 | 3.6 | 19.8 | 6.78 |
| 3 | 129.1 | 5.5 | 185.2 | 67.9 | 0.0858 | 0.4004 | 118.6 | 2.85 | 5.786 | 2.00 | 4.0 | 20.1 | 6.80 |
| 4 | 151.1 | 44.9 | 165.3 | 127.9 | 0.3312 | 0.2285 | 88.5 | 3.32 | 5.803 | 1.75 | 5.4 | 20.1 | 6.78 |
| 5 | 157.0 | 74.1 | 190.5 | 150.5 | -- | 0.1741 | 69.6 | 3.51 | 5.811 | 1.66 | 6.5 | 20.2 | 6.80 |
| 6 | 163.4 | 85.8 | 201.4 | 167.7 | -- | 0.1430 | 72.0 | 3.90 | 5.812 | 1.49 | 6.4 | 20.5 | 6.78 |
| 7 | 168.0 | 106.9 | 195.7 | 194.0 | -- | 0.1678 | 52.0 | 4.40 | 5.842 | 1.33 | 6.3 | 20.8 | 6.82 |
| 8 | 185.0 | 126.0 | 214.0 | 187.0 | -- | 0.1377 | 52.2 | 5.09 | 5.863 | 1.15 | 6.0 | 21.0 | 6.76 |
|  |  |  |  |  |  |  |  |  |  | Average |  | 20.2 | 6.79 |
|  |  |  |  |  |  |  |  |  |  | Standard Deviation |  | 0.54 | 0.047 |
|  |  |  |  |  |  |  |  |  |  | Coefficient of Variation (%) |  | 2.6 | 0.7 |

*$V = 5471 + 231.63H$ (Equation 144)

where $H = 0.1703 + 0.3868 \log Q$ (Equation 146)

Environmental Factors. The important environmental factors were the flow rate and the dissolved oxygen concentration, pH, and temperature of the mixed liquor. The coefficients of variation* for flow rates, temperature, and pH during any run did not exceed 4.0, 5.0, and 1.1 percent, respectively. The dissolved oxygen (DO) was not lower than 1.8 ppm during the course of any run. Therefore, sufficient dissolved oxygen was present and growth was not influenced by DO in these experiments since the values maintained were always above the 0.5 mg/ℓ shown not to be growth limiting (61). Table 10 summarizes the variability of temperature and pH between the runs with a given type of substrate.

Table 10. Variability of Temperature and pH Between the Runs for the Indicated Substrates

| Substrate | Mean of All Runs | | Variability Between Runs | | | |
|---|---|---|---|---|---|---|
| | | | Standard Deviation | | Coefficient of Variation (%) | |
| | Temp(°C) | pH | Temp(°C) | pH | Temp | pH |
| Glucose | 20.5 | 6.76 | 0.43 | 0.06 | 2.1 | 0.8 |
| Galactose | 20.3 | 6.81 | 0.60 | 0.11 | 3.0 | 1.6 |
| Mixed Glucose and Galactose | 20.2 | 6.79 | 0.54 | 0.05 | 2.6 | 0.7 |

Substrate and Organism Concentrations. Figure 40 shows the variability of selected parameters for conditions of steady states with glucose as the substrate. The coefficient of variation of steady state glucose appeared to be a function of the detention time: it was highest

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$$^{*}\text{coefficient of variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

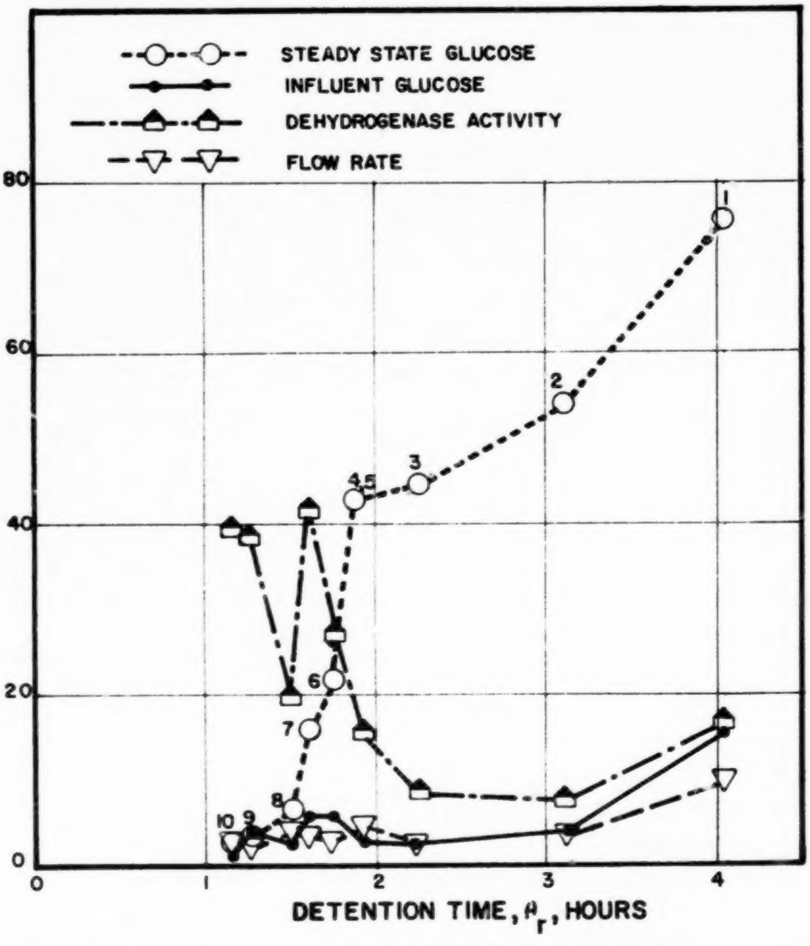

FIGURE 40. VARIABILITY OF INFLUENT AND STEADY STATE GLUCOSE CONCENTRATIONS, DEHYDROGENASE ACTIVITY AND FLOW RATE AS FUNCTIONS OF DETENTION TIME.

at the highest detention time, but it decreased sharply as detention time was decreased. The high variability of the reactor glucose concentration at high detention times (Runs 1 through 5) is partly attributable to the relatively low degree of accuracy of the analytical technique used to measure glucose concentrations below 10 ppm. It has been pointed out by several authors that steady state substrate concentrations are more unstable in continuous cultures with heterogeneous populations than in pure cultures (222,398,399,400). Therefore, as the heterogeneity of the population decreases with the increase in dilution rates in continuous mixed cultures, it is logical to expect (as shown in Figure 40) that the instability (as measured in terms of variability from the mean) will also decrease with increase in dilution rates. Thus a high degree of stability of steady state glucose concentration could be attained at the lower detention times; the variabilities at the two lowest detention times were of the same order of magnitude as the variabilities of the flow rates at these detention times. The low variabilities of influent glucose concentrations and flows were not dependent on detention times and could only be due to experimental inconsistencies and equipment performance errors.

Although data from the glucose runs are discussed herein to illustrate the variability of steady state and its dependence on detention time, similar conclusions could also be drawn from the data of the galactose and glucose-galactose runs.

The change in the variation of the substrate concentration under conditions of steady states of the mixed continuous cultures can be illustrated by considering the growth rate curves of seven different species (see Figure 41) constituting a hypothetical mixed culture. If

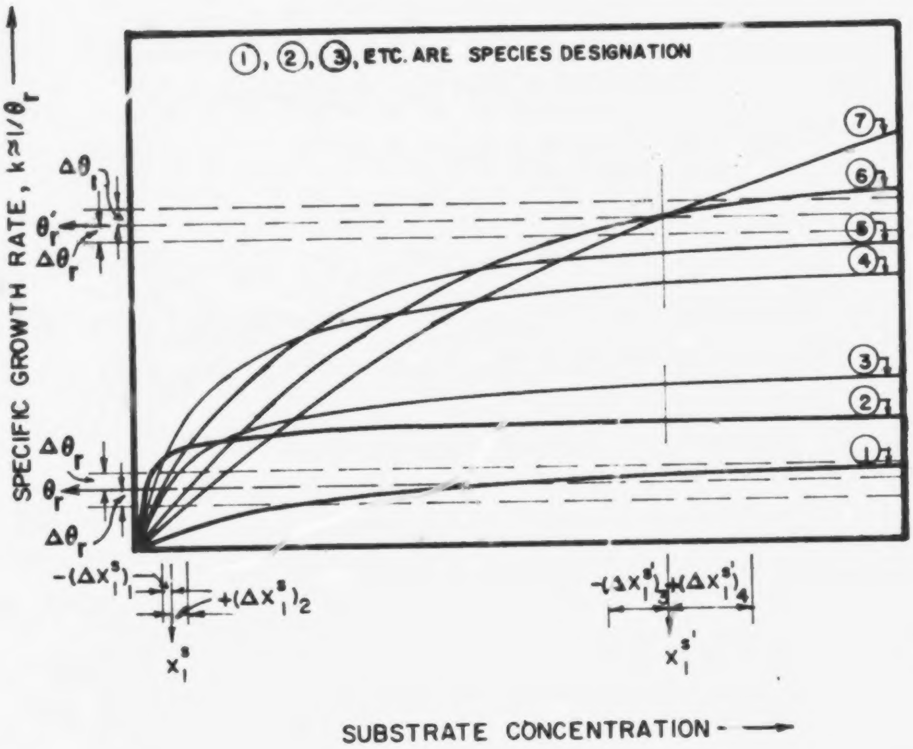

FIGURE 41. THE SPECIFIC GROWTH RATE CURVES OF SEVEN MICROBIAL SPECIES CONSTITUTING A HYPOTHETICAL MIXED CULTURE.

this hypothetical reactor is operated at a high detention time, $\theta_r$, Species 2 predominates by virtue of its capability to grow faster at low concentrations of substrate, albeit all seven species can survive at this detention time. Clearly, Species 2 would prevail at the highest population density relative to others as it grows most efficiently at low dilution rates and substrate concentrations. If it is assumed that the steady state concentration of substrate at the detention time of $\theta_r$ is $X_1^s$, then for any finite positive deviation, $\Delta\theta_r$, of the detention time, Species 2 will have increased advantage over the other species with the result that the steady state substrate concentration will be lowered by $(\Delta X_1^s)_1$. On the other hand, for any negative deviation of the detention time, all species other than Species 2 gain in their population densities with the result that there is an upswing in the steady state substrate concentration by an amount of $(\Delta X_1^s)_2$. However, due to the hyperbolic nature of the growth rate curves, $(\Delta X_1^s)_2$ would be greater than $(\Delta X_1^s)_1$ even though $\theta_r$ deviated by the same amount, $\Delta\theta_r$. If the detention time is now decreased to $\theta_r'$, Species 1 through 5 will be washed out as the dilution rate $(1/\theta_r')$ exceeds the maximum specific growth rates of these species. Thus, the heterogeneity of the bacterial population will decrease markedly due to increase in the dilution rate. Fluctuation of the detention time by an amount $\Delta\theta_r$ will cause fluctuations in the steady state substrate concentration by amounts $-(\Delta X_1^{s'})_3$ and $+(\Delta X_1^{s'})_4$. The magnitudes of the deviations due to the nature of the basic relationships are such that the oscillations $(\Delta X_1^s)_1/X_1^s$ and $(\Delta X_1^s)_2/\Delta X_1^s$ at lower dilution rates are greater than those, namely $(\Delta X_1^{s'})_3/X_1^{s'}$ and $(\Delta X_1^{s'})_4/X_1^{s'}$. at higher dilution rates (see Figure 41). It may therefore be concluded that, in heterogeneous continuous cultures, unstable and oscillatory steady states are almost inevitable; furthermore, the instability increases with increasing detention times due to the prevalance of higher degrees of heterogeneity. Some investigators (222,253,398,401) have implied that steady states in heterogeneous cultures are extremely oscillatory and have suggested the use of frequency response analysis to fit the oscillatory steady states. The preceding discussion served to indicate that steady states in heterogeneous cultures need not be highly oscillatory, especially at higher dilution rates if slime growth is controlled. Most investigators did not control slime growth which was responsible for the high levels of oscillations of steady state substrate and organism concentrations.

## Continuous Culture Studies with Glucose and Galactose

The steady state data from the experimental runs with glucose and galactose were used for the determination of the growth kinetic constants of the organisms on each substrate. The data also furnished information regarding the variation of the physiological capability of the cultures with shifting of the detention time.

Glucose Studies. The specific death rate constant, $k'$, the true yield coefficient, Y, the coefficient of energy of maintenance, m, the substrate utilization factor, $U_s$, the maximum specific growth rate constant, $k^m$, and the saturation constant, K, were determined from the intercepts and slopes of the linear regression lines of Equations 93, 60A, 63, and 64B, respectively. The values of the dependent and the independent variables of the different linear functions are presented in Table 11 for the various detention times employed. Figures 42 and 43

Table 11. Data for Determination of Growth Constants for Glucose Grown Cultures

| Run No. | Detention Time $\theta_r$ (hrs) | $\frac{X_1^{o\,t}}{X_1^o}$ | $\frac{X_o^g - X_1^g}{X_1^o} = \frac{1}{Y^{o\,a}}$ | Observed Yield Coefficients | | $\frac{\theta_r}{1+k'\theta_r}$ (hr) | $\frac{10^3}{X_1^g}$ $((mg/\ell)^{-1})$ | $\frac{1}{\theta_r}+k' = k$ $(hr^{-1})$ |
|---|---|---|---|---|---|---|---|---|
| | | | | Active Solids $Y^{o\,a}$ | Total Solids $Y^{o\,t} = \frac{X_1^{o\,t}}{X_o^g - X_1^g}$ | | | |
| 1 | 2 | 3 | 5 | 6 | 7 | 9 | 10 | 12 |
| 1 | 4.05 | 1.4630 | 2.92 | 0.343 | 0.500 | 2.7555 | 357.143 | 0.363 |
| 2 | 3.12 | 1.5494 | 2.72 | 0.368 | 0.570 | 2.2909 | 322.581 | 0.437 |
| 3 | 2.26 | 1.7636 | 3.09 | 0.324 | 0.570 | 1.7905 | 400.000 | 0.559 |
| 4 | 1.94 | 1.1806 | 1.98 | 0.505 | 0.598 | 1.5837 | 172.414 | 0.632 |
| 5 | 1.94 | 0.9093 | 1.66 | 0.602 | 0.549 | 1.5837 | 172.414 | 0.632 |
| 6 | 1.76 | 1.3692 | 2.18 | 0.459 | 0.629 | 1.4616 | 24.331 | 0.684 |
| 7 | 1.62 | 1.2348 | 1.96 | 0.510 | 0.631 | 1.3638 | 15.576 | 0.733 |
| 8 | 1.52 | 1.3083 | 2.03 | 0.493 | 0.645 | 1.2922 | 11.710 | 0.774 |
| 9 | 1.28 | 1.2836 | 1.85 | 0.541 | 0.695 | 1.1145 | 11.416 | 0.897 |
| 10 | 1.17 | -- | 1.33 | 0.752 | -- | 1.0302 | 7.587 | 0.971 |

Note: 1. Figures in columns 3, 5, 7, and 10 are calculated from data in Table 7.

2. Specific death rate, $k' = 0.116$/hr from Figure 42.

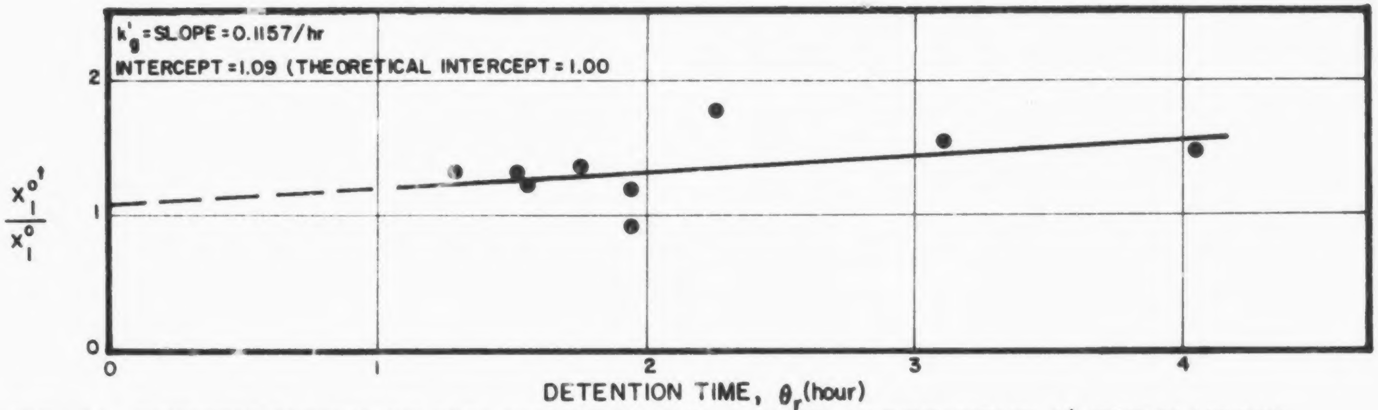

FIGURE 42. DETERMINATION OF THE SPECIFIC DEATH RATE COEFFICIENT, k', FOR GLUCOSE GROWN CULTURES.

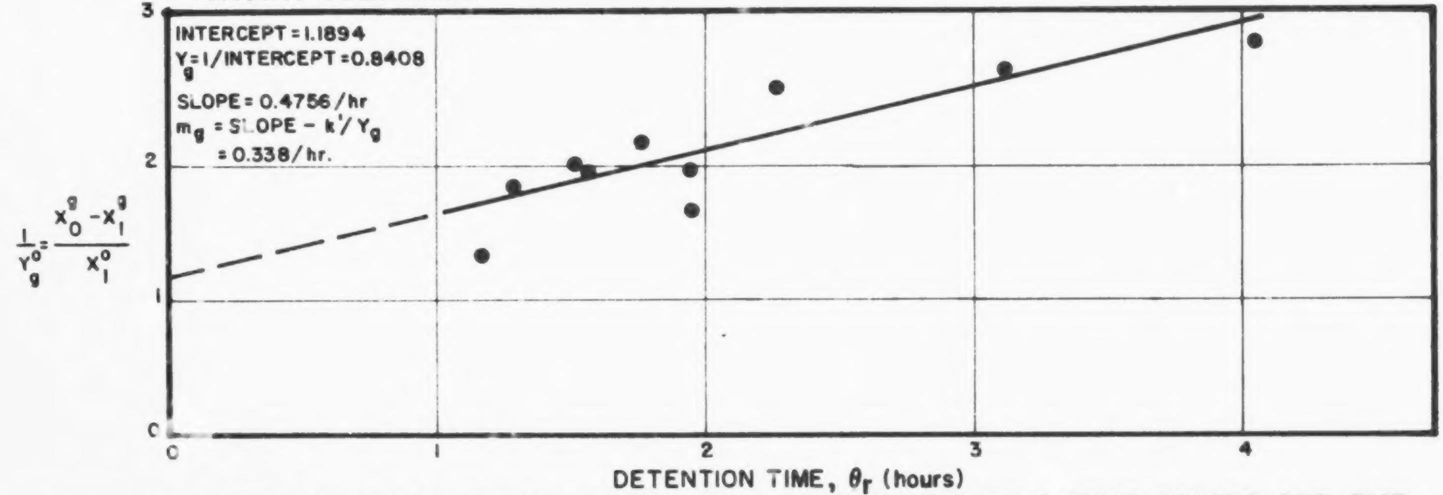

FIGURE 43. DETERMINATION OF THE TRUE YIELD COEFFICIENT OF ACTIVE SOLIDS AND THE MAINTENANCE COEFFICIENT FOR GLUCOSE GROWN CULTURES.

show the regression lines of Equations 93 and 60A, and the values of $k_g'$, $Y_g$, $U_g$, and $m_g$[*] were determined to be 0.116/hr, 0.841, 1.19, and 0.338/hr, respectively.

The plot of data points in Figure 44A indicated that a curve (shown as dashed line) apparently could fit the data better than the linear regression Line 1 (solid line). The data thus emphasized the important fact that, as detention time was decreased[**], the intercept decreased meaning that the maximum specific growth rate of the culture, $k_g^m$, increased. The slope of the dashed curve increased with the decrease of detention time which, coupled with the increase in $k_g^m$, meant that the saturation constant, $K_g$, increased with the decrease in detention time. It appears, therefore, that, at low detention times (high dilution rates), the composition of the culture apparently changed from one dominated by species with low $k_g^m$ and $K_g$ to one in which species characterized by appreciably higher values of $k_g^m$ and $K_g$ dominated. Experimental results obtained from continuous culture runs with galactose and mixtures of glucose and galactose (see Figures 48 and 56) also indicated similar shifts in the taxonomic makeup of the population at lower detention times. The aspect of population shifts with shift in detention time is discussed in a following section. Since the aggregate microbial populations at detention times below 1.96 hours appeared to be characterized by higher values of $k^m$ and K, it was decided to use two linear regression lines to represent the data: (a) regression Line 3 (Figure 44B) to represent the data collected

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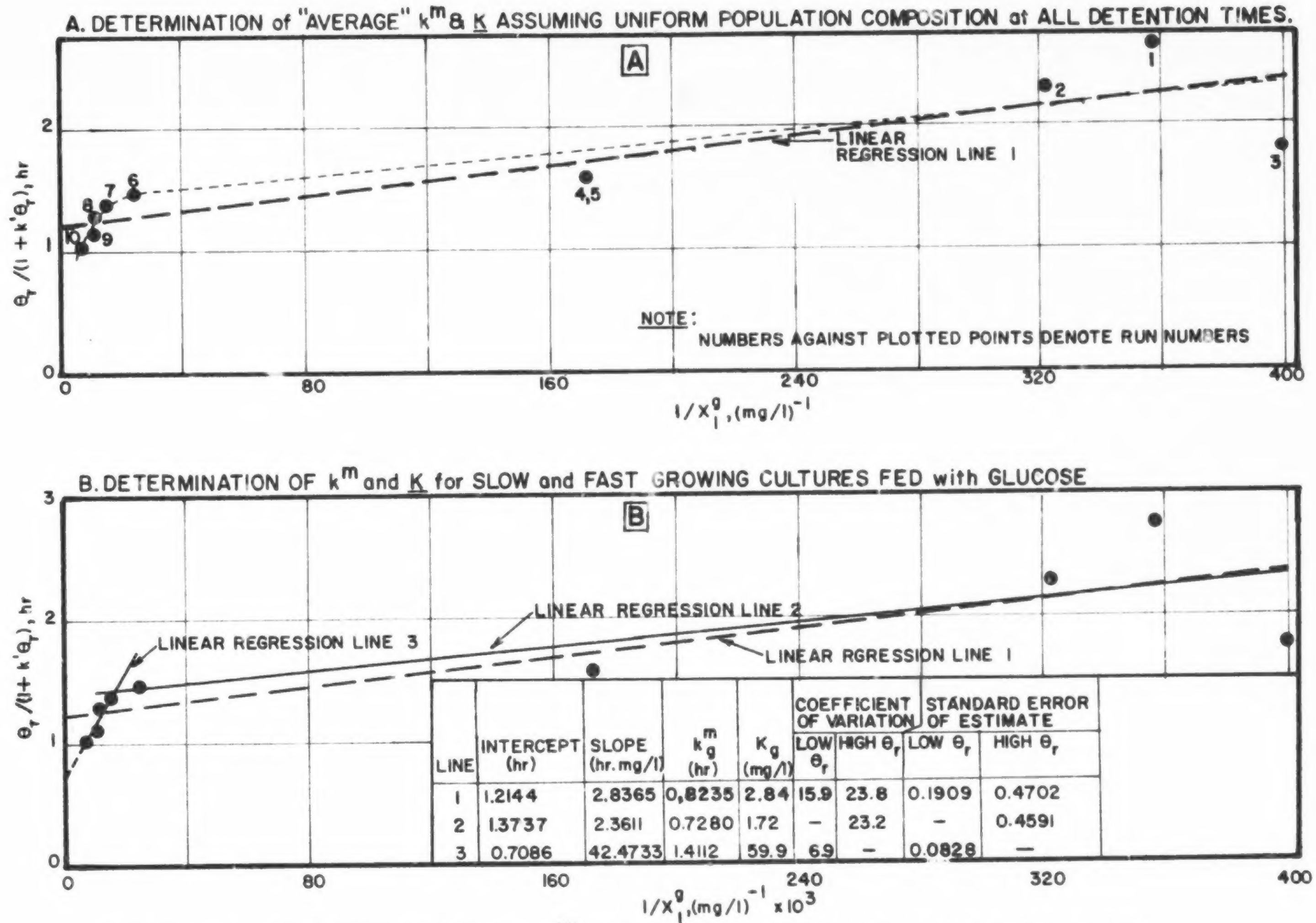
[*] The subscript, g, is used to indicate that the parameters k´, Y, U, and m are for the glucose grown cultures.

[**] It should be noted that increase or decrease of detention times is tantamount to increase or decrease of $\theta_r/(1+k'\theta_r)$ of Figure 44A.



A. DETERMINATION of "AVERAGE" $k^m$ & $\underline{K}$ ASSUMING UNIFORM POPULATION COMPOSITION at ALL DETENTION TIMES.

B. DETERMINATION OF $k^m$ and $\underline{K}$ for SLOW and FAST GROWING CULTURES FED with GLUCOSE

NOTE: NUMBERS AGAINST PLOTTED POINTS DENOTE RUN NUMBERS

| LINE | INTERCEPT (hr) | SLOPE (hr. mg/l) | $k_g^m$ (hr) | $K_g$ (mg/l) | COEFFICIENT OF VARIATION |  | STANDARD ERROR OF ESTIMATE |  |
|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | LOW $\theta_r$ | HIGH $\theta_r$ | LOW $\theta_r$ | HIGH $\theta_r$ |
| 1 | 1.2144 | 2.8365 | 0,8235 | 2.84 | 15.9 | 23.8 | 0.1909 | 0.4702 |
| 2 | 1.3737 | 2.3611 | 0.7280 | 1.72 | – | 23.2 | – | 0.4591 |
| 3 | 0.7086 | 42.4733 | 1.4112 | 59.9 | 6.9 | – | 0.0828 | – |

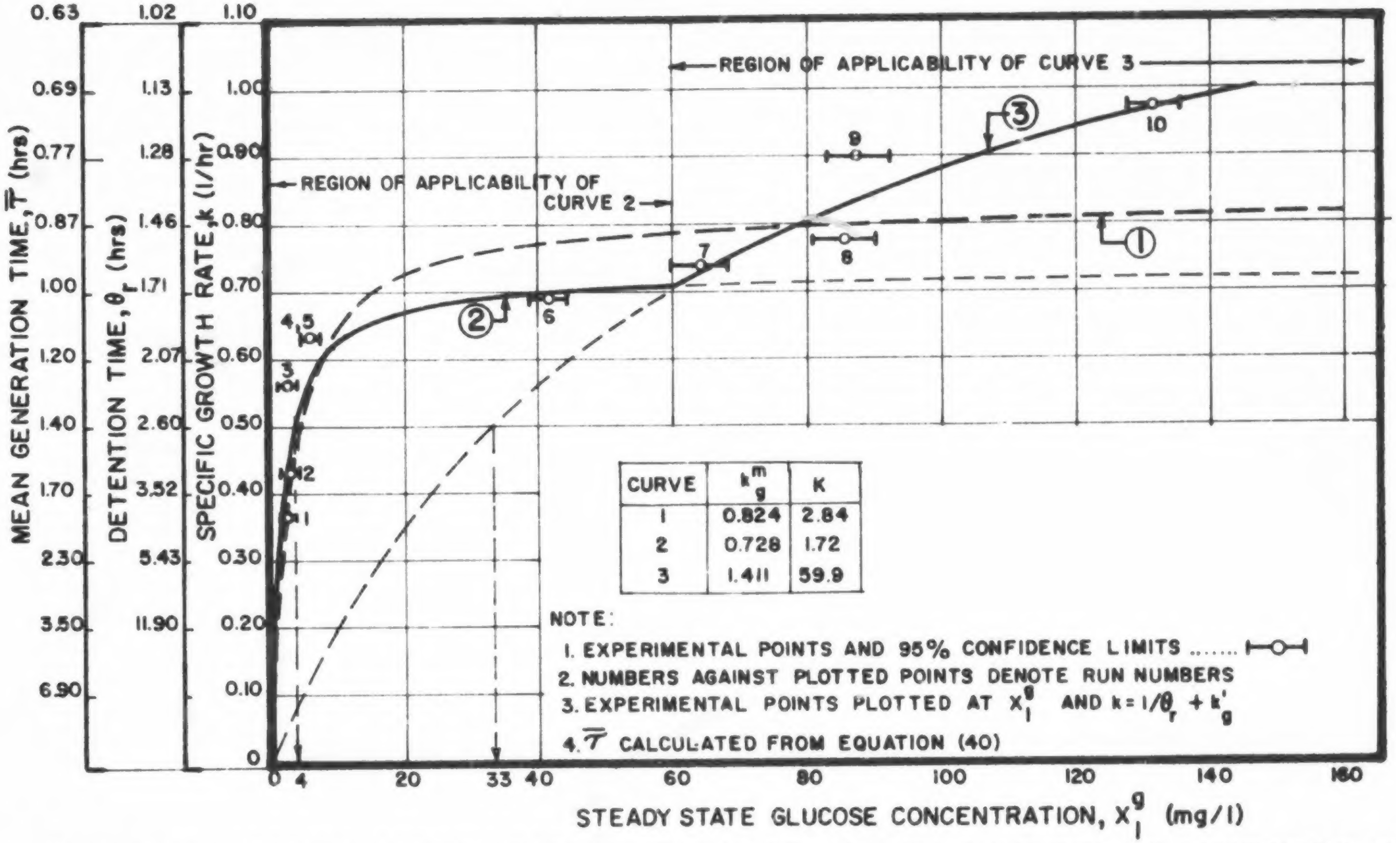
FIGURE 44. DETERMINATION OF $k^m$ AND $\underline{K}$ FOR GLUCOSE GROWN CULTURES.

at lower detention times, and (b) Line 2 (Figure 44B) to represent data at higher detention times. From comparisons of the standard errors of estimate as noted in the table of Figure 44, it was concluded that two regression lines yielding two sets of $k_g^m$ and $K_g$ provided a better stochastic description of the observations than a single regression line.

Curves 2 and 3 of Figure 45 are the calculated growth rate curves for the slow and fast growing populations at the higher and lower detention times when glucose served as the sole substrate. The solid curve fits the observations better then Curve 1 defined by the "average" $k_g^m$ and $K_g$ computed from regression Line 1 of Figure 44A. The computed values of $k_g^m$ and $K_g$ for Curves 1 and 2 of Figure 45 are compared in Table 12 with the values of these constants reported by other investigators.

At very low detention times (below about 1.25 hours), cultures grown on glucose produced about 65 mg/ℓ of acetic acid as determined by chromatography (F & M Scientific 700 Laboratory Chromatograph, Hewlett-Packard, Pasadena, California). Volatile acid production in high rate aerobic processes has been observed by Pirt (235), Chain and Mateles (222), and Maxon and Johnson (391). The phenomenon of volatile acid production by aerobic organisms may be explained by the postulate that, at very high catabolic rates, the oxidative enzymes may become saturated and the glycolytic enzymes having volatile acids as end products may become operative. The production of organic acids points to the limitations of the high rate process.

Galactose Studies. The data presented in Table 13 were used for the determination of the regression lines illustrated in Figures 46, 47, and 48. The specific death rate constant, $k'_{ga}$, the true yield coefficient,



| CURVE | $k_g^m$ | K |
|---|---|---|
| 1 | 0.824 | 2.84 |
| 2 | 0.728 | 1.72 |
| 3 | 1.411 | 59.9 |

NOTE:
1. EXPERIMENTAL POINTS AND 95% CONFIDENCE LIMITS ....... ⊢○⊣
2. NUMBERS AGAINST PLOTTED POINTS DENOTE RUN NUMBERS
3. EXPERIMENTAL POINTS PLOTTED AT $X_1^g$ AND $k = 1/\theta_r + k_g'$
4. $\overline{T}$ CALCULATED FROM EQUATION (40)

FIGURE 45. SPECIFIC GROWTH RATES OF GLUCOSE GROWN CULTURES AS FUNCTIONS OF STEADY STATE GLUCOSE CONCENTRATIONS.

Table 12. Comparison of Values of the Maximum Specific Growth Rate Constant and the Saturation Constant Reported by Various Investigators for Glucose Grown Cultures

| Investigator | Organism | Type of Culture | $k_g^m$ ($hr^{-1}$) | $K_g$ (mg/ℓ) |
|---|---|---|---|---|
| 1. Monod (7, 38) | *E. coli* | Batch | 1.35 | 3.96 |
| | *E. coli* | Batch | 1.09 | |
| 2. Rao (402) | *E. coli* | Continuous | -- | 3.78 |
| 3. Baidya, et al. (134) | *K. aerogenes* | Batch | 0.83 | -- |
| 4. Harte and Webb (135) | *K. aerogenes* | Batch | 1.19 | -- |
| 5. Schulze (64) | *E. coli* | Batch | 0.68 | -- |
| 6. Schulze (65) | *E. coli* | Continuous | 1.03 | 95.4 |
| 7. Marlar (221) | Heterogeneous | Batch | 0.34 | 4.7 to 8.0 |
| 8. This study | Heterogeneous* | Continuous | 0.73 | 1.7 |
| | Heterogeneous** | Continuous | 1.41 | 59.9 |
| | Heterogeneous | Batch | 0.40 | -- |

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*Slow growers at detention times above 1.7 hours.

**Fast growers at detention times below 1.7 hours.

Table 13. Data for Determination of Growth Constants for Galactose Grown Cultures

| Run No. | Detention Time $\theta_r$ (hrs) | $\frac{X_1^{o\,t}}{X_1^o}$ | $\frac{X_o^{ga}-X_1^{ga}}{X_1^o} = \frac{1}{Y^{oa}}$ | Observed Yield Coefficients | | $\frac{\theta_r}{1+k'\theta_r}$ (hr) | $\frac{10^3}{X_1^{ga}}$ (mg/$\ell^{-1}$) | $k = \frac{1}{\theta_r}+k'$ (hr$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | | | | Active Solids $Y^{oa}$ | Total Solids $Y^{o\,t} = \frac{X_1^{o\,t}}{X_o^{ga}-X_1^{ga}}$ | | | |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | 5.92 | 1.0778 | 1.5820 | 0.6321 | 0.681 | 5.0991 | 232.558 | 0.196 |
| 2 | 5.83 | 1.3306 | 2.5766 | 0.3881 | 0.516 | 5.0319 | 250.000 | 0.199 |
| 3 | 3.81 | 1.1027 | 1.9384 | 0.5159 | 0.569 | 3.4523 | 526.316 | 0.290 |
| 4 | 3.26 | | -- | -- | 0.593 | 2.9944 | 15.898 | 0.334 |
| 5 | 2.96 | 1.5000 | 2.4475 | 0.4086 | 0.613 | 2.7395 | 11.403 | 0.365 |
| 6 | 2.84 | 1.0162 | 1.7267 | 0.5791 | 0.589 | 2.6365 | 37.313 | 0.379 |
| 7 | 2.51 | 1.0751 | 2.1528 | 0.4645 | 0.585 | 2.3495 | 9.930 | 0.416 |
| 8 | 2.11 | -- | -- | | | 1.9955 | 8.606 | 0.501 |
| 9 | 2.06 | 1.1688 | 2.6197 | 0.3817 | 0.5630 | 1.9508 | 41.667 | 0.513 |
| 10 | 1.94 | | 1.3249 | 0.7548 | | 1.8427 | 5.787 | 0.543 |
| 11 | 1.65 | 0.9743 | 2.2367 | 0.4471 | 0.4656 | 1.5791 | 7.468 | 0.633 |

Note: 1. Figures in columns 3, 4, 6, and 8 are calculated from data in Table 8.

2. Specific death rate, $k' = 0.0272$/hr from Figure 46.

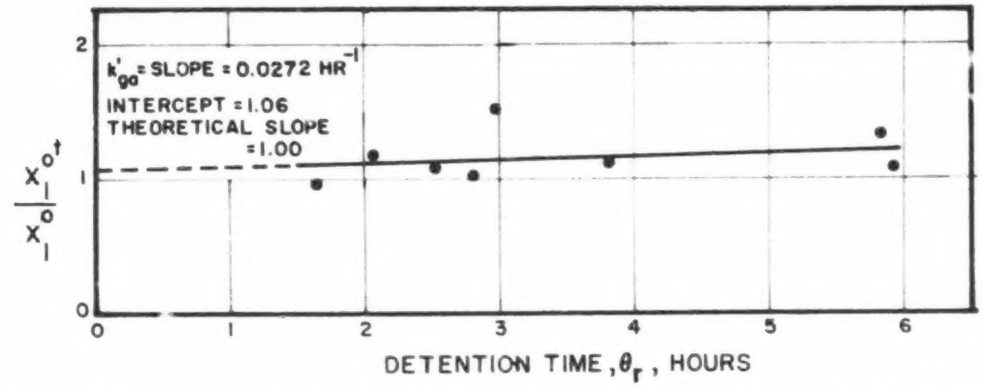


FIGURE 46. DETERMINATION OF THE SPECIFIC DEATH RATE COEFFICIENT, $k'_{ga}$ FOR GALACTOSE GROWN CULTURES.

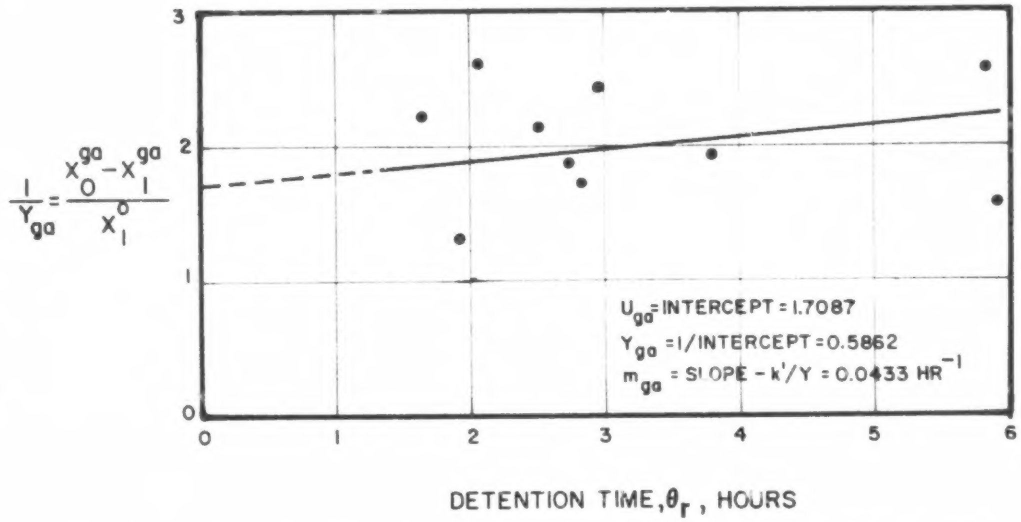
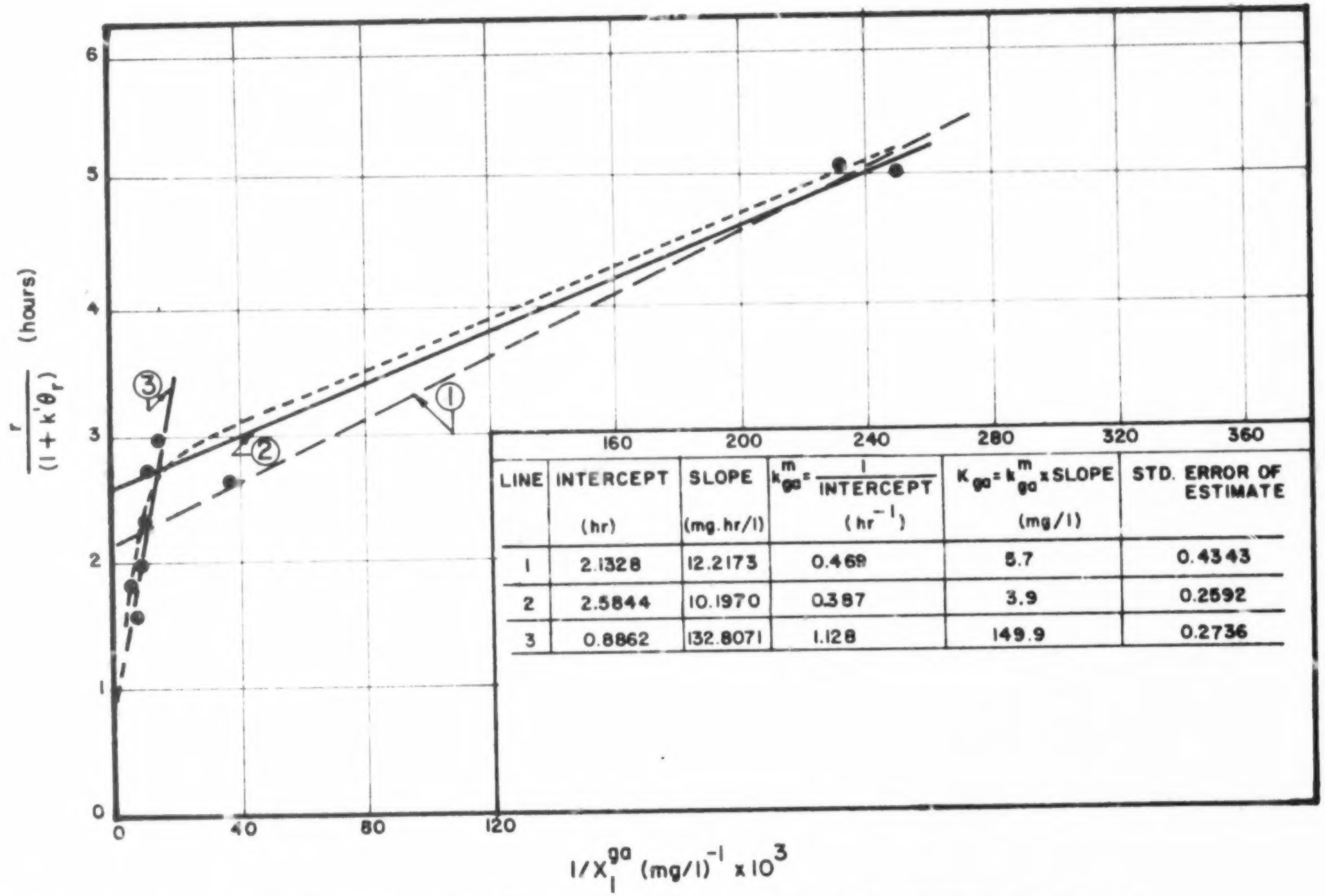


FIGURE 47. DETERMINATION OF TRUE YIELD COEFFICIENT AND MAINTENANCE COEFFICIENT FOR GALACTOSE GROWN CULTURES.



| LINE | INTERCEPT (hr) | SLOPE (mg.hr/l) | $k_{ga}^m = \frac{1}{\text{INTERCEPT}}$ $(hr^{-1})$ | $K_{ga} = k_{ga}^m \times \text{SLOPE}$ (mg/l) | STD. ERROR OF ESTIMATE |
|---|---|---|---|---|---|
| 1 | 2.1328 | 12.2173 | 0.469 | 5.7 | 0.4343 |
| 2 | 2.5844 | 10.1970 | 0.387 | 3.9 | 0.2592 |
| 3 | 0.8862 | 132.8071 | 1.128 | 149.9 | 0.2736 |

FIGURE 48. DETERMINATION OF $k^m$ AND K FOR GALACTOSE GROWN CULTURES

$Y_{ga}$, the substrate utilization factor, $U_{ga}$, and the coefficient of energy of maintenance, $m_{ga}$[*], were calculated to be 0.027/hr, 0.586, 1.709, and 0.0433/hr, respectively. Figure 48 very much resembles Figure 44A, and the kinetic characteristics of the galactose cultures at lower detention times were clearly defined by considerably higher values of $k^m_{ga}$ and $K_{ga}$. Thus, the heterogeneous populations prevailing at the two ranges of detention times demonstrated different kinetic characteristics as summarized below:

Populations prevalent at $\theta_r > 3.0$; $k^m_{ga} = 0.387$/hr, $K_{ga} = 3.9$ mg/ℓ

Populations prevalent at $\theta_r < 3.0$; $k^m_{ga} = 1.128$/hr, $K_{ga} = 149.9$ mg/ℓ

Figure 49 shows the growth rate curves defined by these two sets of constants.

Comparison of the Growth Kinetic Properties of Cultures Grown on Glucose and Galactose. One of the objectives of this research was to investigate if the growth kinetic constants of each individual substrate were indicative of its fate when subjected to microbial degradation along with other competing substrates. In order to predict the probable role of glucose and galactose in controlling microbial growth on mixed glucose-galactose, it is necessary to critically compare the growth kinetic properties of cultures grown on either substrate as the sole carbon and energy source. The growth kinetic constants for the heterogeneous cultures grown on glucose and galactose are compared in Table 14.

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[*] The subscript, ga, is used to indicate that the parameters $k'$, Y, U, and m are for the galactose grown cultures.

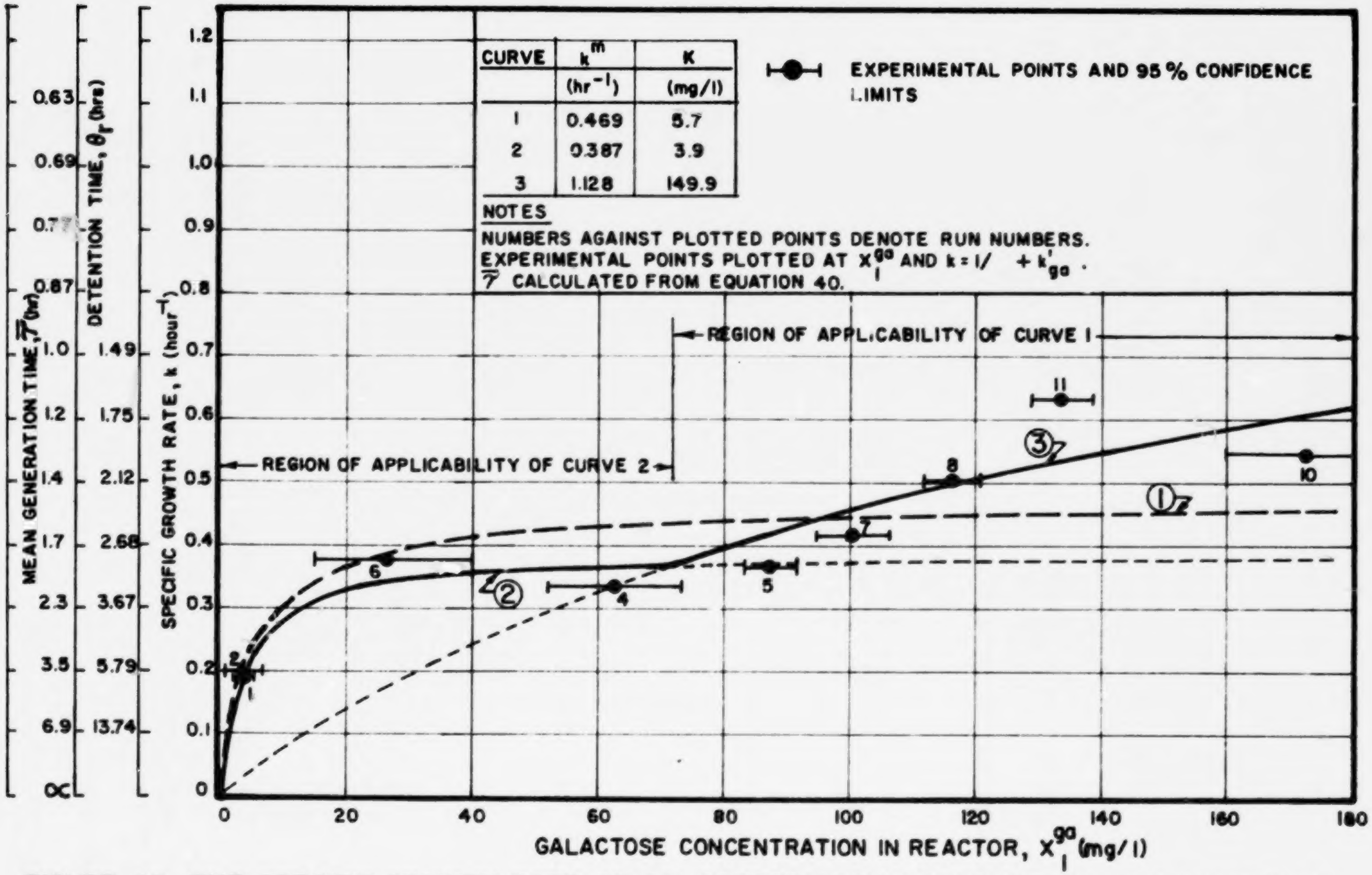


FIGURE 49. THE SPECIFIC GROWTH RATE OF GALACTOSE GROWN CULTURES AS A FUNCTION OF STEADY STATE GALACTOSE CONCENTRATION.

Table 14. Comparison of Growth Kinetic Constants of Glucose and Galactose Grown Cultures

| Substrate | $k'$ $\text{hr}^{-1}$ | Y | $U_s$ | m $\text{hr}^{-1}$ | High Detention Time | | | Low Detention Time | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $k^m$ $\text{hr}^{-1}$ | $\tau$ min hr | K mg/ℓ | $k^m$ $\text{hr}^{-1}$ | $\tau$ min hr | K mg/ℓ |
| Glucose | 0.116 | 0.841 | 1.189 | 0.338 | 0.728 | 0.952 | 1.7 | 1.411 | 0.491 | 59.9 |
| Galactose | 0.027 | 0.586 | 1.709 | 0.043 | 0.387 | 0.179 | 3.9 | 1.128 | 0.615 | 149.9 |

The specific death rate constant, $k'$, of cultures grown on galactose was considerably lower than that for cultures proliferating on glucose. As shown in Figure 50, the galactose grown cells were exposed to a much higher substrate concentration than the cells utilizing glucose. Substrate assimilations and total solids concentrations were, in general, higher at all detention times for the glucose substrate. Consequently, glucose grown cells were confronted with a high degree of physical crowding of cells, as well as higher concentrations of metabolic waste products. Lower substrate tension, crowding, and higher concentrations of waste products are possible causative effects for the observed high specific death rate of the glucose grown cultures. The data therefore supported the arguments advanced by some authors (13,42,53,81) that death rate is a direct function of concentration of toxic metabolites and solids concentration, and an inverse function of extracellular substrate concentration.

Some remarks regarding the technique of measurement of the specific death rate constant are appropriate at this point. The values obtained by the chosen procedure did not seem to be unrealistic. The

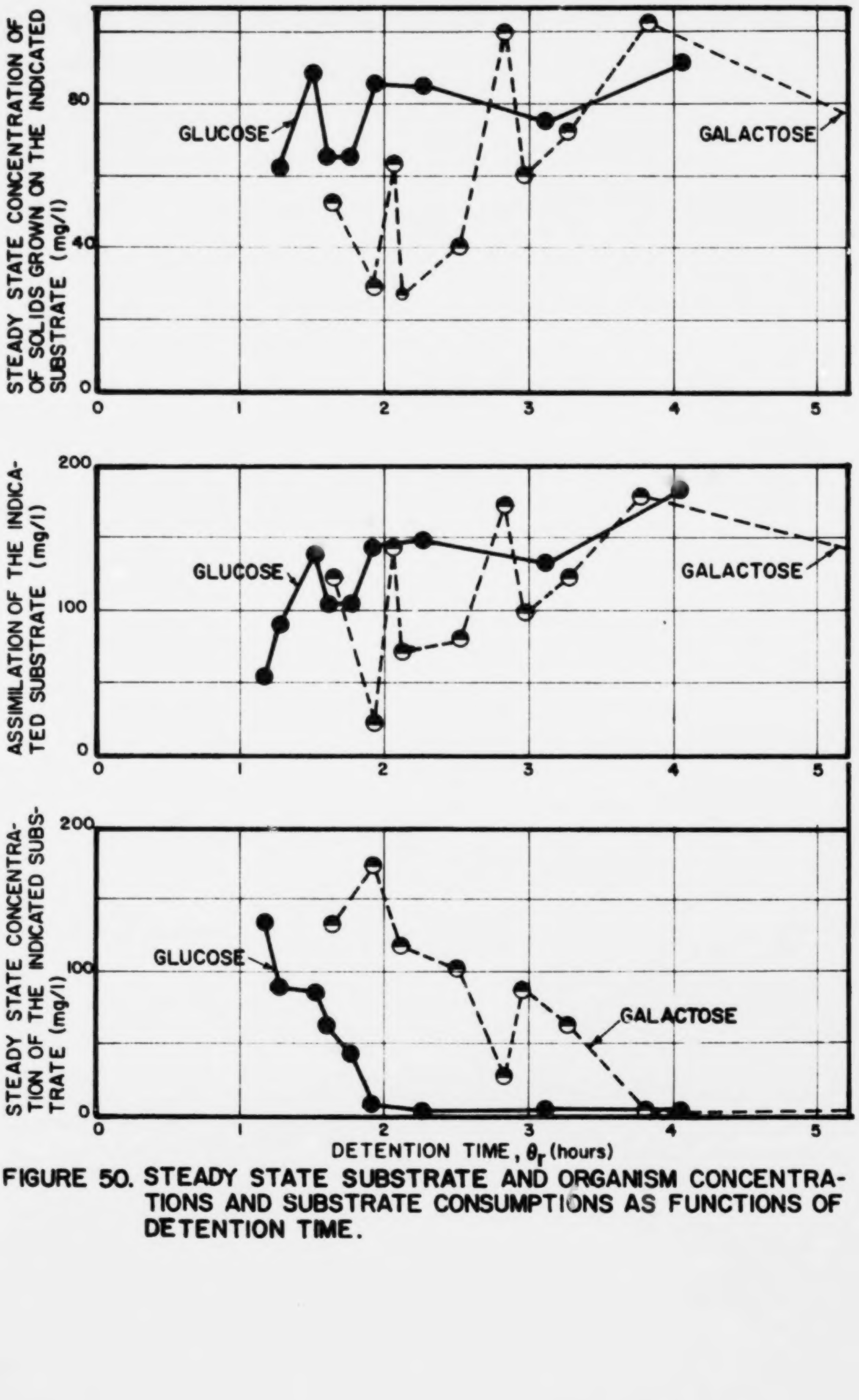


FIGURE 50. STEADY STATE SUBSTRATE AND ORGANISM CONCENTRATIONS AND SUBSTRATE CONSUMPTIONS AS FUNCTIONS OF DETENTION TIME.

variation of $\underline{k}'$ for the glucose and galactose grown cultures could be appropriately attributed to variation in culture density, anticipated concentration levels of toxic metabolites and extracellular concentrations of the substrate. Furthermore, the linearity of the plots of Figures 42 and 46 as dictated by the theory and the fact that the intercepts (1.09 and 1.06) of the linear plots closely approximated the theoretical intercept of 1.00, furnished evidence that the adopted technique was capable of providing a good estimate of this constant.

A comparison of the values of substrate utilization factors, $U_s$, and maintenance coefficient, m, for the glucose and galactose grown cultures revealed that more galactose had to be diverted for synthesis of unit quantity of biomass than was required with glucose. As a result, the efficiency of the synthetic process (as reflected in the lower value of the yield factor) was lower when galactose served as the carbon source. The consequence of the apparent inefficiency of the synthetic process was that less galactose could be diverted for furnishing the energy of maintenance through biological oxidation. On the other hand, a relatively lower quantity of glucose (1.189 mg of glucose compared to 1.709 mg of galactose) was needed for the synthesis of unit of quantity of biomass; as a result, more glucose was available for energy of maintenance. The lower substrate utilization factor for glucose resulted in a higher true growth yield and a higher maintenance coefficient. There was little doubt that the growth supporting ability of glucose was substantially higher than that of galactose. In addition, the coefficient of energy of maintenance of the dominants apparently varied directly with the yield factor and the maximum specific growth rate.

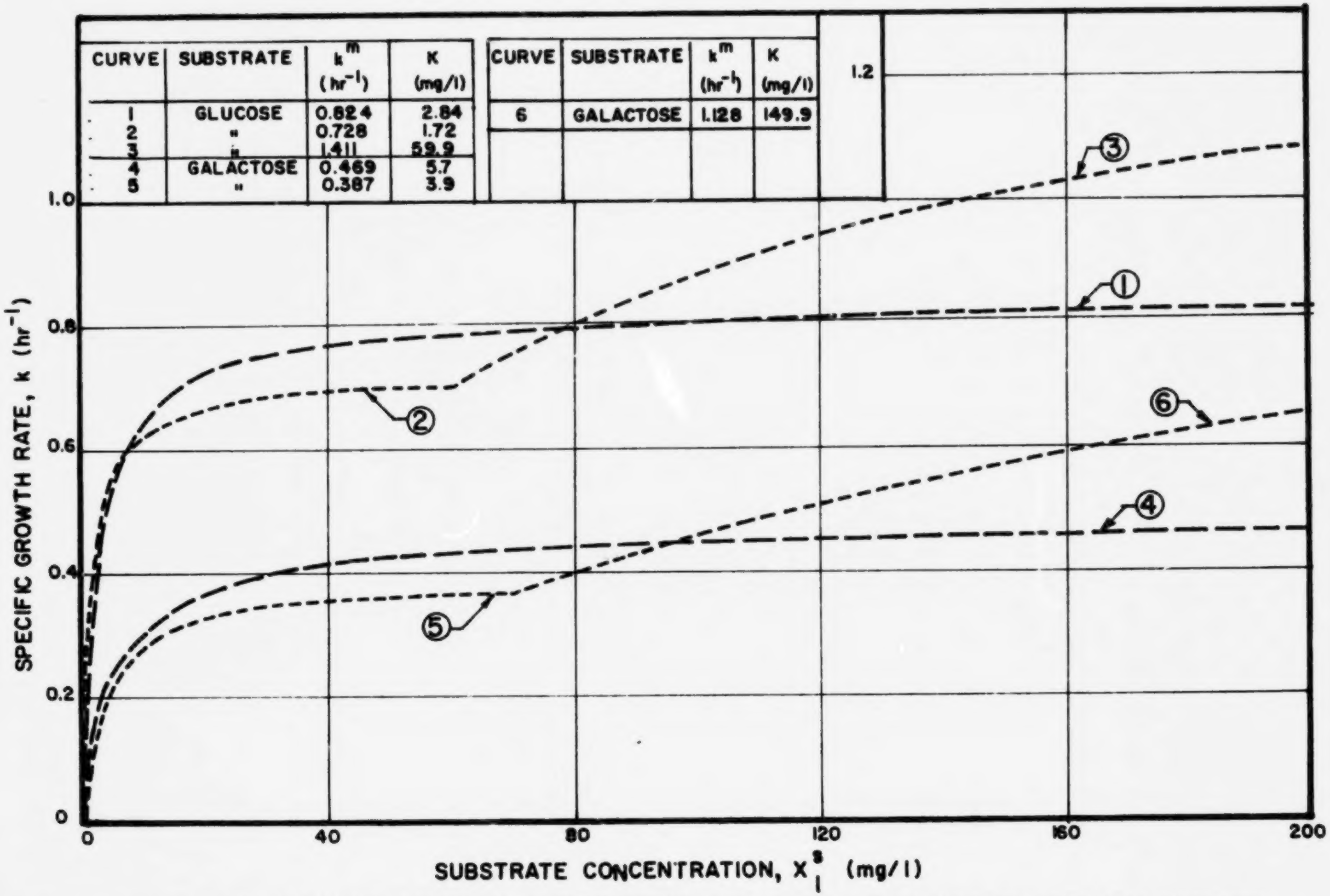
The yield coefficients on glucose and galactose were substantially different although both sugars undergo the same enthalpy change in oxidation. Thus, the contention of McKinney (114,115) that yield factor is dependent on the enthalpy change of substrate oxidation is questionable.

The yield coefficients on glucose and galactose were 0.40 and 0.31 for batch cultures (see Chapter VI) compared to 0.84 and 0.59, respectively (see Table 14), for continuous cultures. Such differences in cell yields from batch and continuous cultures were also observed by Pirt (235). As has been pointed out by numerous authors (253,226,59, 225,240,321), inefficient metabolism and lower growth yields in a closed growth system like that of a batch culture are attributable to continuous change of oxygen and substrate concentration, pH, gradual buildup of concentration of toxic metabolic products, and heterogeneous age distribution of the cells of the culture.

The maintenance coefficient for growth on glucose (0.338) appears to be high, but maintenance coefficients of 0.473 and 0.300 for growth of _A. cloacae_ on glucose and of _Lipolytic bacterium_ on glycerol, respectively, have been reported (235,403). Only limited data on maintenance coefficients for heterogeneous cultures have been reported in the literature. The value of the maintenance coefficient for growth on galactose (0.0433) was of the same order of magnitude as some of the reported values of 0.076 (_A. aerogenes_, glucose)(59), 0.094 (_A. cloacae_, glucose)(235), and 0.055 (_E. coli_, glucose)(62-65).

It should be recognized that the substrate utilized for energy of maintenance is oxidized to $CO_2$ and $H_2O$ in aerobic systems as was experimentally proved by Marr, *et al.* (66). It can be concluded, therefore, that, compared to galactose-fed cultures, glucose grown cells oxidized a higher fraction of the assimilated substrate for maintenance.

The higher maximum specific growth rates of glucose grown cultures (see Figure 51) indicate the higher efficiency of glucose as a carbon and energy source. It had been postulated by Monod (38) and also shown in Chapter IV that $\underline{k}^{m}$ is related to the maximum velocity of the *master* or *pacemaker* enzyme of the enzyme system involved in the breakdown of the substrate. After permeation and catalytic actions by the three galactose enzymes, galactose catabolism proceeds *via* the same route as followed for breakdown of glucose-6-phosphate which is the first catabolite from glucose (see Figure 4). In view of these facts, it is apparent that the lower $\underline{k}^{m}$ for galactose could have been caused either because the galactose permease or any of the three galactose enzymes became the *pacemaker* enzyme whose maximum velocity must have been lower than the maximum velocity of the pacemaker enzyme in glucose metabolism. Horecker, *et al.* (20) have concluded that, during bacterial metabolism of galactose, transport across the cell membrane by the permease system was the rate limiting step. Kipnis, *et al.* (404) also concluded that permease transport controls galactose uptake in animal tissues. Cohn and Monod (152) and Pollock (153) also pointed out that permease transport was the rate limiting step in many cases. It is therefore quite possible that in the systems studied, permease transport was the growth controlling step in the galactose grown cells. The Michaelis constant for the galactose permease system of *E. coli* has been reported to be 1.8 (20) and 0.2 (272) mg/ℓ measured as external concentration of



| CURVE | SUBSTRATE | $k^m$ ($hr^{-1}$) | K (mg/l) |
|---|---|---|---|
| 1 | GLUCOSE | 0.824 | 2.84 |
| 2 | " | 0.728 | 1.72 |
| 3 | " | 1.411 | 59.9 |
| 4 | GALACTOSE | 0.469 | 5.7 |
| 5 | " | 0.387 | 3.9 |

| CURVE | SUBSTRATE | $k^m$ ($hr^{-1}$) | K (mg/l) |
|---|---|---|---|
| 6 | GALACTOSE | 1.128 | 149.9 |

FIGURE 51. COMPARISON OF GROWTH SUPPORTING ABILITIES OF GLUCOSE AND GALACTOSE.

galactose. A saturation constant of four mg/ℓ for galactose compares favorably with the reported Michaelis constant considering that $K = K_M/f$ (see Equation 51, Chapter IV) and f is a factor less than 1. Thus the value of $K_{ga}$ reported herein also substantiates the contention that the galactose permease system, in all probability, became the rate controlling enzymatic step in galactose metabolism. Since the reciprocal of the saturation constant is a measure of affinity of the cells for the substrate, it is evident, based on the experimental data, that the cultures had a relatively higher affinity for glucose as a substrate for growth.

Comparison of the maximum specific growth rates attained on the two sugars at high and low detention times indicated that relative to glucose, galactose was not as inefficient a substrate for fast growing species as it was for the slow growers at high detention times.

The important conclusion which can be drawn from comparisons of the growth constants of glucose and galactose is that, of the two substrates, glucose allows lower minimum generation times and higher growth yields which in turn better ensure survival by more rapid multiplication and total growth. It is not difficult to imagine, therefore, that a given bacterial population would prefer glucose as the growth controlling substrate when confronted with both glucose and galactose, so long as the former can sustain the highest achievable growth rate. Under such circumstances, physiological adaptation to galactose by formation of the galactose enzymes may be delayed until a point is reached where the depleted glucose concentration allows a growth rate also allowed by the heretofore undepleted concentration of galactose. The result of such a scheme of substrate utilization would be the occurrence of diauxie. Based on the differences between the maximum specific growth rates of glucose and galactose ($k^m_g - k^m_{ga}$) at low and high detention times (see Figure 51), it may be expected that phasic uptake of glucose and galactose would be more favored by the slow growers under almost all circumstances. On the other hand, the fast growers may encounter environments in which the galactose concentration may be so high relative to glucose concentration that both substrates can allow equally short generation times and under such circumstances the organisms need not resort to sparing of the galactose and no diauxie would be anticipated. Additional continuous culture experiments with mixtures of glucose and galactose were deemed necessary to verify these preceding postulates.

## Continuous Culture Studies with Glucose-Galactose Mixtures

The purposes of extending the experiments with mixed sugars were to: (a) observe and record the growth rates at which galactose may be spared as a carbon and energy source, and (b) ascertain if the growth kinetic constants determined for the individual sugar should also describe its uptake rate from the mixture. All environmental factors (pH, temperature, concentrations of DO and nutrients, etc.) were necessarily maintained at the same levels as in cases of the runs with individual sugars.

Active Solids Concentrations and the Specific Death Rate. Preliminary plots of specific growth rates, which are approximately equal to $1/\theta_r$ (see Equation 73), as a function of substrate concentrations provided ample evidence that the experimental data could be described by the Monod growth rate Equation 52. The procedure for determination of the growth constants, as already outlined in the previous sections, requires the use of active solids concentrations, $X_1^o$, and the specific death rate constant, $k'$. However, the active solids concentrations determined from the measured dehydrogenase activities were very low, so much so that these could only be accounted for by unrealistic yield factors. Furthermore, the concentrations of active solids so determined were unexpectedly lower than the measured total solids concentration, as shown in Figure 52. If it is assumed for the moment that the correlation between the dehydrogenase activity and active solids was valid for the organisms grown on glucose-galactose mixtures, then the difference between the observed total solids concentrations and the computed active solids concentrations could only be attributed to inactive solids and cell debris caused by death. The specific death rates at various detention times, as computed from Equation 93 and shown in Figure 52, were obviously unrealistic as these exceeded 1.0 at most detention times. It was apparent, therefore, that the established correlation between dehydrogenase activities and active solids concentrations did not apply to solids grown on mixed sugars. Consequently, it was decided to compute active solids concentration from Equation 93 using the data on total dry solids concentration and a value of specific death rate constant, $k'$, estimated in a manner described below.

Based on the established fact that $\underline{k'}$ depended on the solids and substrate concentrations as well as on the amount of total substrate consumed, a realistic estimate of $\underline{k'}$ could be determined from a comparative study of these levels in the glucose and the glucose-galactose cultures. From Figure 53A and 53B it appeared that the latter cultures

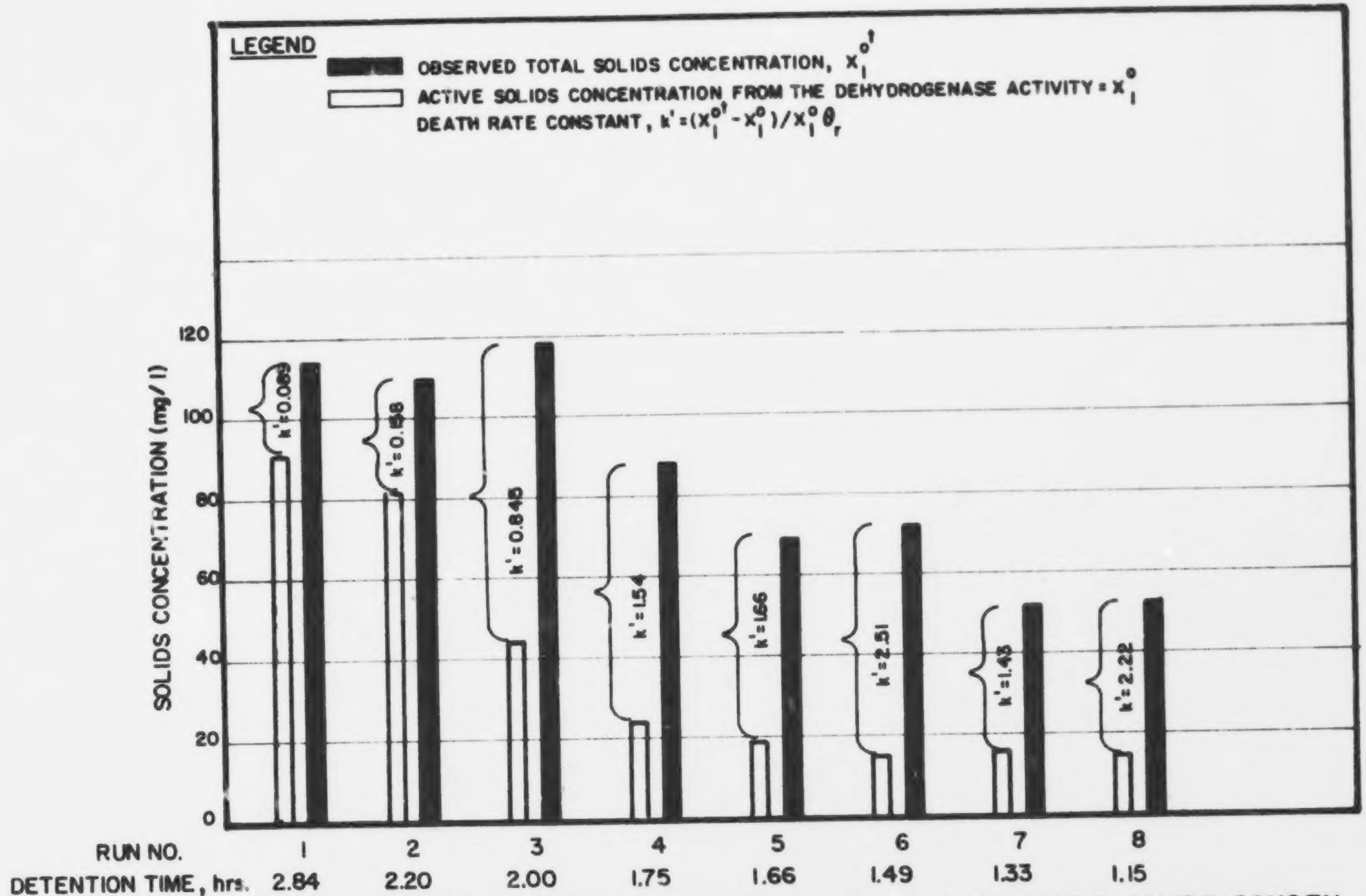


FIGURE 52. COMPARISON OF TOTAL SOLIDS CONCENTRATIONS WITH APPARENT SOLIDS CONCENTRATIONS AS INDICATED BY DEHYDROGENASE ACTIVITIES.

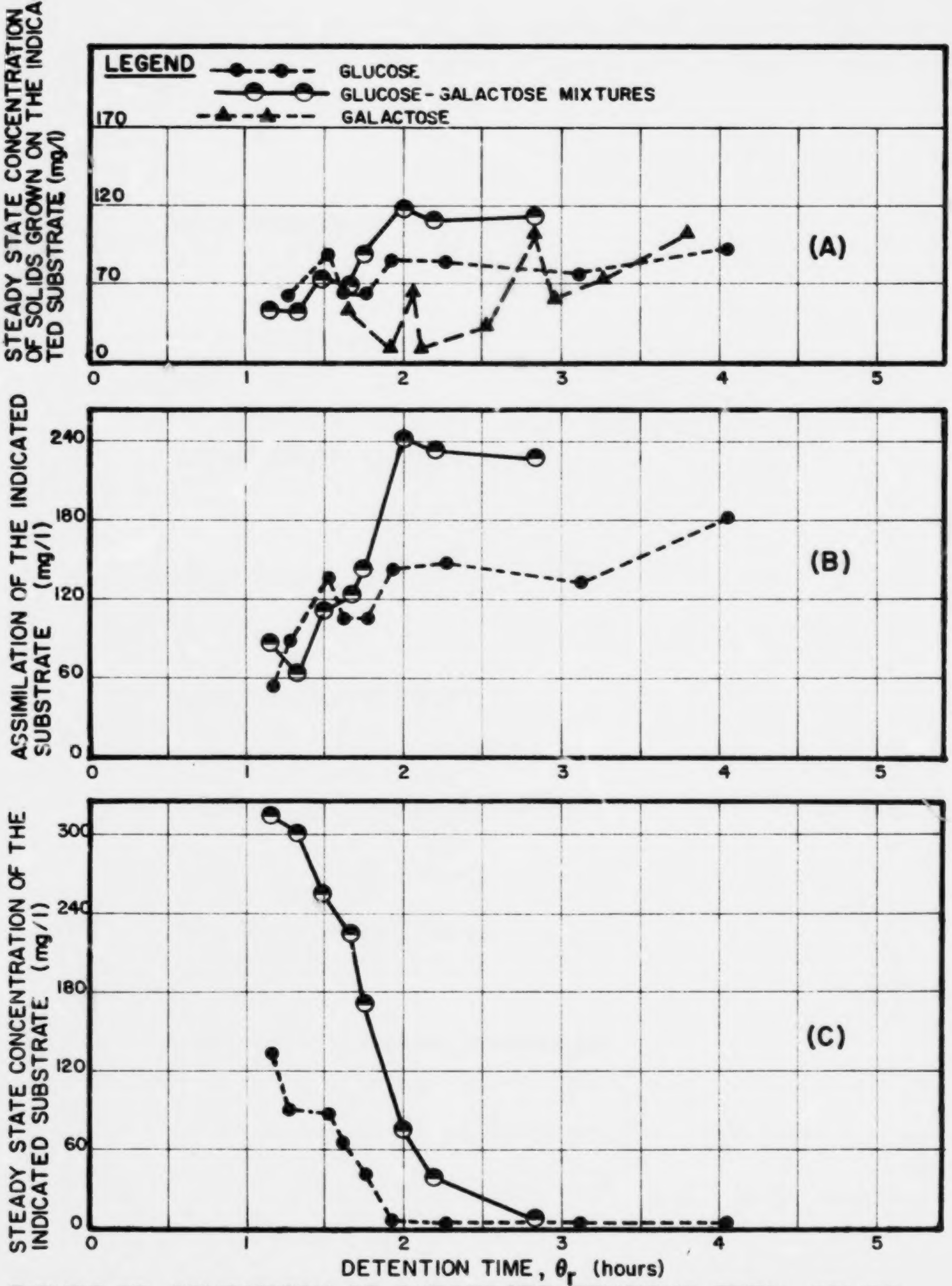


FIGURE 53. COMPARISON OF SUBSTRATE TENSIONS, TOTAL SOLIDS CONCENTRATIONS AND TOTAL SUBSTRATE CONSUMPTIONS IN CULTURES GROWN ON GLUCOSE AND MIXED GLUCOSE-GALACTOSE.

encountered more crowding and toxic metabolites than the former. This suggested that cultures grown on glucose-galactose mixtures might have experienced a death rate higher than that of the glucose cultures (in which $k' = 0.116/\text{hr}$). However, the higher levels of total substrate concentrations also shown on Figure 53C could conceivably offset the adverse effects of higher levels of crowding and toxic metabolites. Therefore, it can be anticipated that the specific death rates of the cultures grown on glucose and glucose-galactose mixtures could not have been very different. On this basis, a specific death rate constant of 0.116/hr was assumed for the cultures fed with mixed sugars. Inasmuch as the value of $\underline{k'}$ for cells using mixed substrates could not be determined by a more sophisticated technique, the assumed value of 0.116/hr was not considered so different from the true value as to significantly affect the analyses which made use of this constant.

Having chosen the value of $\underline{k'}$, the active solids concentrations of Table 15 could be determined from Equation 93.

Determination of Substrate Utilization Factors, True Yield Factor, and Coefficients of Energy of Maintenance. In order to determine the amounts of glucose and galactose utilized for growth and energy of maintenance, the following substrate balances analogous to Equation 58 were performed:

$$V \frac{dX_i^{ga'}}{d\theta} = X_o^{ga'} Q - X_i^{ga'} Q - kX_i^o V U'_{ga} - m'_{ga} X_i^o V \qquad (154)$$

where $X_i^{ga'}$ = galactose concentration in reactor in the presence of glucose

$U'_{ga}$ = galactose assimilated for growth in terms of milligrams of galactose used for synthesis of a milligram of biomass

$m'_{ga}$ = galactose utilized for energy of maintenance in terms of milligrams of galactose used per milligram of biomass per hour.

At steady state, Equation 154 reduces to

$$\frac{X_0^{ga}-X_1^{ga'}}{\theta_r} = X_1^o[kU'_{ga} + m'_{ga}] \tag{155}$$

From the organism balance it can be shown, in a manner similar to the organism balance of Equation 54 (see Chapter IV), that

$$k = \frac{1}{\theta_r} + k' \tag{56}$$

Substituting Equation 56 into Equation 155 and rearranging

$$\frac{X_0^{ga}-X_1^{ga'}}{X_1^o} = U'_{ga} + \theta_r(k'U'_{ga} + m'_{ga}) \tag{156}$$

Similarly from a glucose balance

$$\frac{X_0^{g}-X_1^{g}}{X_1^o} = U'_{g} + \theta_r(k'U'_{g} + m'_{g}) \tag{157}$$

where $U'_g$ = glucose assimilated for growth per unit quantity of biomass produced in terms of mg of glucose utilized for synthesis of one mg of biomass, and

Table 15. Data for Determination of $U_g'$, $U_{ga}'$, $m_g'$, and $m_{ga}'$ for Cultures Grown on Glucose-Galactose Mixtures

| Run No. | Detention Time, $\theta_r$ (hr) | Viable Solids*, $X_i^o$ (mg/ℓ) | Substrate Utilization per Unit Quantity of Biomass | |
|---|---|---|---|---|
| | | | Glucose $(X_o^g-X_i^g)/X_i^o$ | Galactose $(X_o^{ga}-X_i^{ga'})X_i^o$ |
| 1 | 2 | 3 | 4 | 5 |
| 1 | 2.84 | 85.8 | 1.2909 | 1.3621 |
| 2 | 2.20 | 87.6 | 1.3579 | 1.3259 |
| 3 | 2.00 | 96.3 | 1.2839 | 1.2185 |
| 4 | 1.75 | 73.6 | 1.4436 | 0.5084 |
| 5 | 1.66 | 58.4 | 1.4205 | 0.6854 |
| 6 | 1.49 | 61.4 | 1.2640 | 0.5473 |
| 7 | 1.33 | 45.0 | 1.3563 | 0.0285 |
| 8 | 1.15 | 46.1 | 1.2810 | 0.5862 |

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* $X_i^o = \dfrac{X_i^{o\,t}}{(1+k'\theta_r)}$ , $k' = 0.116$/hr as decided from discussion of Figures 52 and 53.

Note: Figures in columns 3, 4, and 5 are computed from data in Table 9.

$m'_g$ = maintenance coefficient in terms of milligrams of glucose used for supplying energy of maintenance per milligram of biomass per hour.

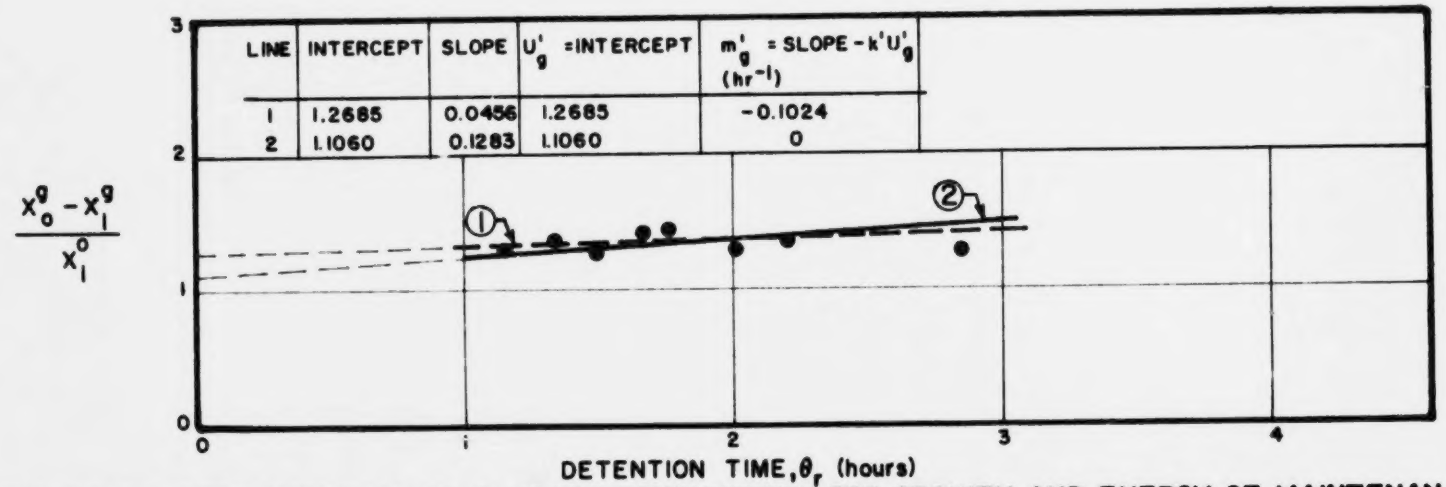
Equations 156 and 157 indicated that the total substrate utilization per unit mass of active organisms is a linear function of the detention time. The substrate utilization factors and the coefficients of energy of maintenance can be determined from the intercepts and the slopes of the straight lines obtained from regression analysis of $(X_o^{ga}-X_1^{ga'})/X_1^o$ or $(X_o^g-X_1^g)/X_1^o$ on $\theta_r$. Figures 54 and 55 show the regression lines (Line 1 in both figures) derived from analyses of data presented in Table 15. These analyses yielded negative values for $m'_g$ and $U'_{ga}$. However, because $m'_g$ and $U'_{ga}$ cannot be less than zero, Equations 156 and 157 may be reduced to the following forms:

$$\frac{X_o^{ga}-X_1^{ga'}}{X_1^o} = m'_{ga}\theta_r \qquad (158)$$

$$\frac{X_o^g-X_1^g}{X_1^o} = U'_g + (k'U'_g)\theta_r \qquad (159)$$

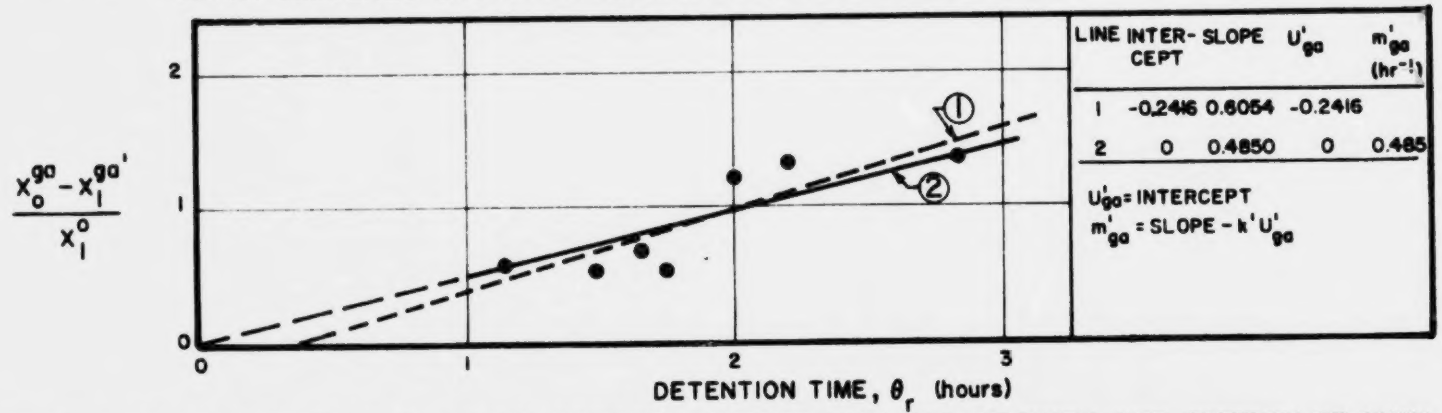
The slopes and intercepts of the straight line functions represented by Equations 156 and 157 were determined from the following normal equations:

$$m'_{ga} = \frac{\sum_{i=1}^{n} \theta_r \left[\frac{X_o^{ga}-X_1^{ga'}}{X_1^o}\right]}{\sum_{i=1}^{n} (\theta_r)^2} \qquad (160)$$



| LINE | INTERCEPT | SLOPE | $U'_g$ =INTERCEPT | $m'_g$ = SLOPE - $k'U'_g$ ($hr^{-1}$) |
|---|---|---|---|---|
| 1 | 1.2685 | 0.0456 | 1.2685 | -0.1024 |
| 2 | 1.1060 | 0.1283 | 1.1060 | 0 |

FIGURE 54. DETERMINATION OF GLUCOSE CONSUMPTION FOR GROWTH AND ENERGY OF MAINTENANCE BY CULTURES GROWN ON GLUCOSE-GALACTOS MIXTURES.



| LINE | INTER-CEPT | SLOPE | $U'_{ga}$ | $m'_{ga}$ ($hr^{-1}$) |
|---|---|---|---|---|
| 1 | -0.2416 | 0.6054 | -0.2416 | |
| 2 | 0 | 0.4850 | 0 | 0.485 |

$U'_{ga}$= INTERCEPT
$m'_{ga}$ = SLOPE - $k'U'_{ga}$

FIGURE 55. DETERMINATION OF GALACTOSE CONSUMPTION FOR GROWTH AND ENERGY OF MAINTENANCE BY CULTURES GROWN ON GLUCOSE-GALACTOSE MIXTURES.

$$U'_g = \frac{\sum_{i=1}^{n} \left[\frac{X_0^g - X_1^g}{X_1^o}\right]}{n + k' \sum_{i=1}^{n} \theta_r} \qquad (161)$$

Equations 160 and 161 can be derived readily for least square fitting of straight lines having zero intercept and a known slope of $k'U'_g$, respectively. $U'_g$ and $m'_{ga}$ were calculated from the intercept and slope, respectively, of the regression lines of Equations 156 and 157 (Line 2 of Figures 54 and 55). Since the assimilated glucose only accounted for growth but not for any part of energy of maintenance, $1/U'_g$ was the true growth yield with respect to glucose.

The interesting conclusion drawn from the foregoing analyses was that the glucose consumption accounted for the total growth, whereas the assimilated galactose appeared to have satisfied all of the requirements of the energy of maintenance. It appears as though the organisms spared glucose, the more efficient growth yielding substrate, from supplying the energy of maintenance. Being the least able of the two to support growth, galactose was used in quantities needed to supply the energy of maintenance. Involvement of an additional NAD in galactose catabolism (see $E_3$-NAD in Figure 4) also makes this substrate preferable to glucose as the energy substrate.* The cells grown on glucose and galactose thus demonstrated a very remarkable capacity in effecting the most efficient allocation of substrates for growth and energy.

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*In the regeneration reaction of a reduced NAD, enough electrochemical energy is released to effect formation of three moles of ATP per mole of $NADH_2$ oxidized. Use of an additional NAD in the galactose pathway would mean the release of additional oxidative energy during galactose degradation.

The growth yield coefficient on glucose in the presence of galactose (0.904) was slightly higher than that obtained on glucose alone (0.841). The cells had the highest energy requirements ($m'_{ga}$ = 0.4850/hr) for maintenance compared to those of the glucose and the galactose grown cells ($m_g$ = 0.338/hr and $m_{ga}$ = 0.0433/hr). The explanations for the very high energy requirements include: (a) more energy of maintenance needed for higher growth yields; (b) more substrate had to be transported across the cell wall which consumed more energy; and finally, (c) additional galactose enzymes had to be synthesized.

Determination of Maximum Specific Growth Rate and Saturation Constant for Cultures Fed with Mixed Sugars. Table 16 presents the data for regression analyses of $\theta_r/(1+k'\theta_r)$ with the reciprocal of glucose and galactose concentrations. The regression lines (Lines A and B) best fitting the experimental points are presented in Figure 56. As with those of Figures 44 and 48 for single substrates, these curves indicated that the intercept decreased and slopes increased (i.e., $k^m$ and K increased) with decreases of detention time. Thus, the culture fed with mixed substrate also showed evidence of shifts in species composition with shifts in the detention time.

From comparisons of the observed steady state glucose concentrations of the glucose runs with the steady state glucose concentrations obtained with the mixed sugar runs (see experimental data points in Figure 57), it appeared that the maximum specific growth rates as well as the saturation constants for glucose were the same in both sets of experiments. This becomes explicit as a result of comparison of two sets

Table 16. Data for Determination of $k^m$ and K for Glucose and Galactose Assimilation by Cultures Grown on Mixtures of Glucose and Galactose

| Run No. | Detention Time, $\theta_r$ (hr) | $\frac{\theta_r}{1+k'\theta_r}$ (hr) | $k = \frac{1}{\theta_r}+k'$ ($hr^{-1}$) | $\frac{10^3}{X_i^{ga}}$ ($(mg/\ell)^{-1}$) | $\frac{10^3}{X_i^{g}}$ ($(mg/\ell)^{-1}$) |
|---|---|---|---|---|---|
| 1 | 2.84 | 2.1363 | 0.468 | 322.581 | 312.500 |
| 2 | 2.20 | 1.7527 | 0.571 | 26.882 | 588.200 |
| 3 | 2.00 | 1.6234 | 0.616 | 14.728 | 181.818 |
| 4 | 1.75 | 1.4547 | 0.687 | 7.819 | 23.310 |
| 5 | 1.66 | 1.3919 | 0.718 | 6.645 | 13.495 |
| 6 | 1.49 | 1.2705 | 0.787 | 5.959 | 11.655 |
| 7 | 1.33 | 1.1522 | 0.868 | 5.155 | 9.355 |
| 8 | 1.15 | 1.0146 | 0.986 | 5.483 | 7.937 |

Note: 1. $k' = 0.116$/hr (see footnote of Table 14).

2. Figures in columns 5 and 6 are calculated from data in Table 8.

| SUBSTRATE | LINE | INTERCEPT (hour) | $k^m$ ($hr^{-1}$) | SLOPE (hr.mg/l) | K (mg/l) | AVERAGE INTERCEPT (hour) | AVERAGE SLOPE (hr.mg/l) | AVERAGE $k^m$ ($hr^{-1}$) | AVERAGE K (mg/l) |
|---|---|---|---|---|---|---|---|---|---|
| GLUCOSE + GALACTOSE | A | 0.5148 | 1.9425 | 65.2632 | 126.74 | 0.6117 | 50.6835 | $\boxed{1.635}$ | $\boxed{82.86}$ |
| GLUCOSE | A' | 0.7086 | 1.4112 | 42.4733 | 59.9 | | | | |
| GLUCOSE + GALACTOSE | B | 1.5782 | 0.6336 | 0.5918 | 0.38 | 1.4646 | 1.2268 | $\boxed{0.683}$ | $\boxed{0.84}$ |
| GLUCOSE | B' | 1.3737 | 0.7280 | 2.3611 | 1.72 | | | | |

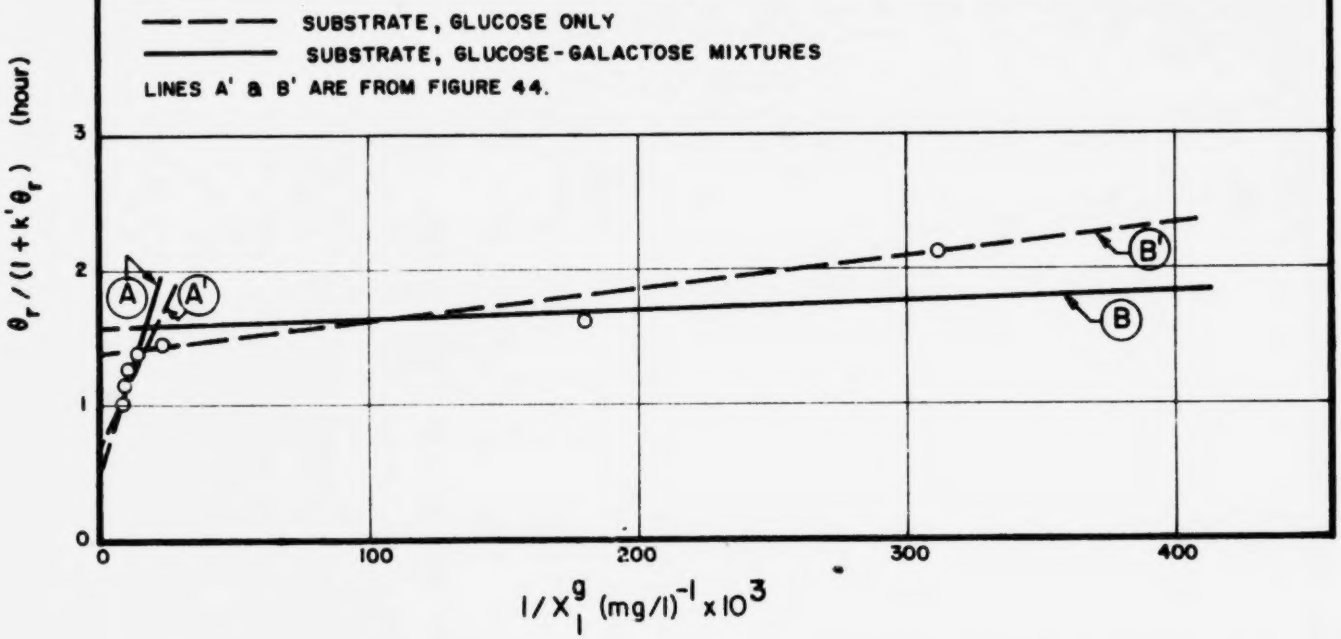


FIGURE 56. DETERMINATION OF $k^m$ AND K FOR GLUCOSE ASSIMILATION BY CULTURES GROWN ON GLUCOSE-GALACTOSE MIXTURES, AND COMPARISON WITH THE PLOT OF FIGURE 44.

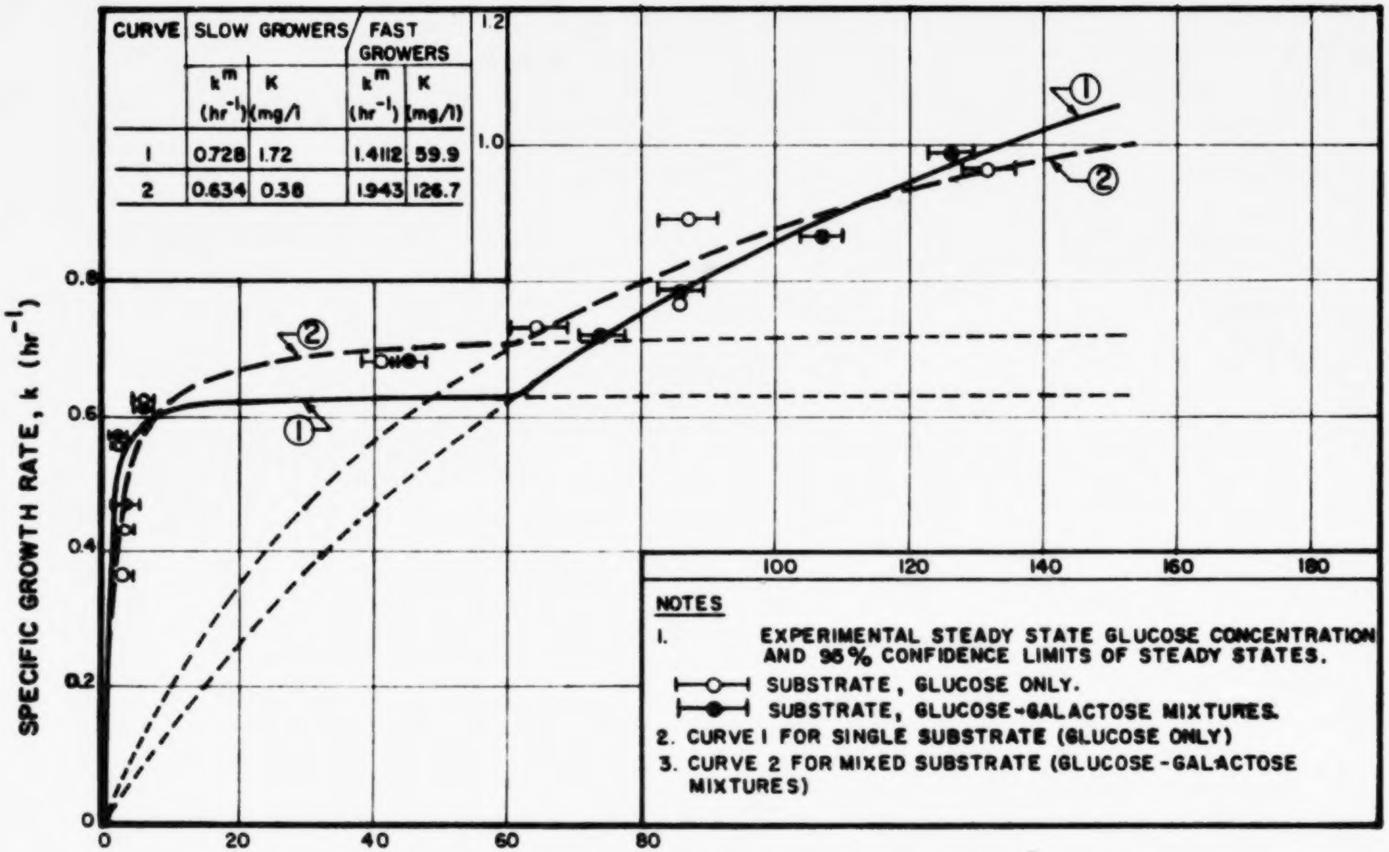
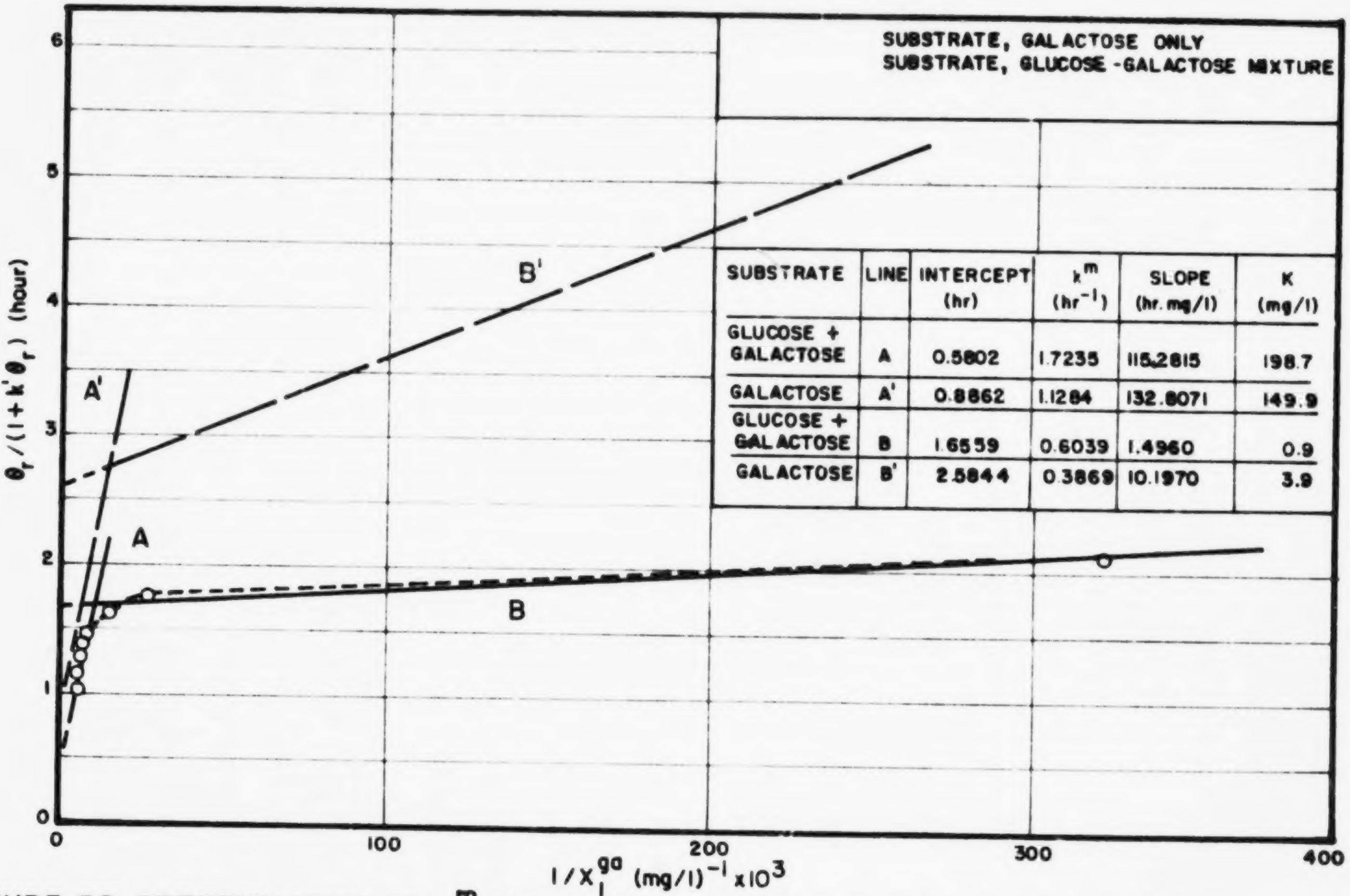


FIGURE 57. COMPARISON OF STEADY STATE GLUCOSE CONCENTRATIONS AT VARIOUS SPECIFIC GROWTH RATES OF CONTINUOUS CULTURES FED WITH GLUCOSE AND GLUCOSE-GALACTOSE MIXTURES.

of regression lines in Figure 56. Statistical tests (371) of the hypothesis that the regression Line B and B' as well as A and A' (Figure 56) were identical, led to the decision that the slopes and intercepts of the two lines of each pair (AA' and BB') did not differ significantly at the five percent level of significance. The average values of $k^m$ and K for microbial uptake of glucose by slow and fast growing dominants, regardless of the presence of galactose, were calculated from the pooled estimates of the mean intercept and slope of each pair of lines. The hyperbolic versions of the linearized forms of Figure 56 are presented in Figure 57. The average values of $k^m$ and K for glucose uptake (see table in Figure 56) were used to compute the mean Curves 1A and 1B of Figure 60 where specific growth rates were plotted as functions of glucose concentrations.

Treatment of the steady state concentrations of galactose in the same manner as outlined for glucose resulted in the regression Lines A and B of Figure 58 and Curve 2 of Figure 59. Regression Lines A' and B' of Figure 58 and the specific growth rate Curve 1 of Figure 59 were derived from the steady state experiments in which galactose was the only substrate. Visual comparison of Curves 1 and 2 of Figure 59 clearly showed that, at any selected detention time (or specific growth rate), the presence of glucose caused substantial decrease in the steady state concentrations of galactose. Statistically, regression Lines A and B differed significantly from Lines A' and B' (Figure 58), respectively, at the five percent level of significance.

It may be well to summarize the salient aspects of the experiment with mixed sugars at this point. In the first place, glucose and galactose



| SUBSTRATE | LINE | INTERCEPT (hr) | $k^m$ $(hr^{-1})$ | SLOPE (hr. mg/l) | K (mg/l) |
|---|---|---|---|---|---|
| GLUCOSE + GALACTOSE | A | 0.5802 | 1.7235 | 115.2815 | 198.7 |
| GALACTOSE | A' | 0.8862 | 1.1284 | 132.8071 | 149.9 |
| GLUCOSE + GALACTOSE | B | 1.6559 | 0.6039 | 1.4960 | 0.9 |
| GALACTOSE | B' | 2.5844 | 0.3869 | 10.1970 | 3.9 |

FIGURE 58. DETERMINATION OF $k^m$ AND K FOR GALACTOSE ASSIMILATION BY CULTURES GROWN ON GLUCOSE-GALACTOSE MIXTURES, AND COMPARISON WITH THE PLOT OF FIGURE 48.

| SUBSTRATE: GALACTOSE, CURVE 1 | | | SUBSTRATE: GLUCOSE-GALACTOSE MIXTURES, CURVE 2 | |
|---|---|---|---|---|
| SLOW GROWERS | $k_{ga}^{m}$ | $K_{ga}$ | $k_{ga}^{m'} = g^{m}$ | $K'_{ga} = G$ |
| SLOW GROWERS | 0.3869 $hr^{-1}$ | 3.9 mg/l | 0.6039 $hr^{-1}$ | 0.9 mg/l |
| FAST GROWERS | 1.1284 $hr^{-1}$ | 149.9 mg/l | 1.7235 $hr^{-1}$ | 198.7 mg/l |

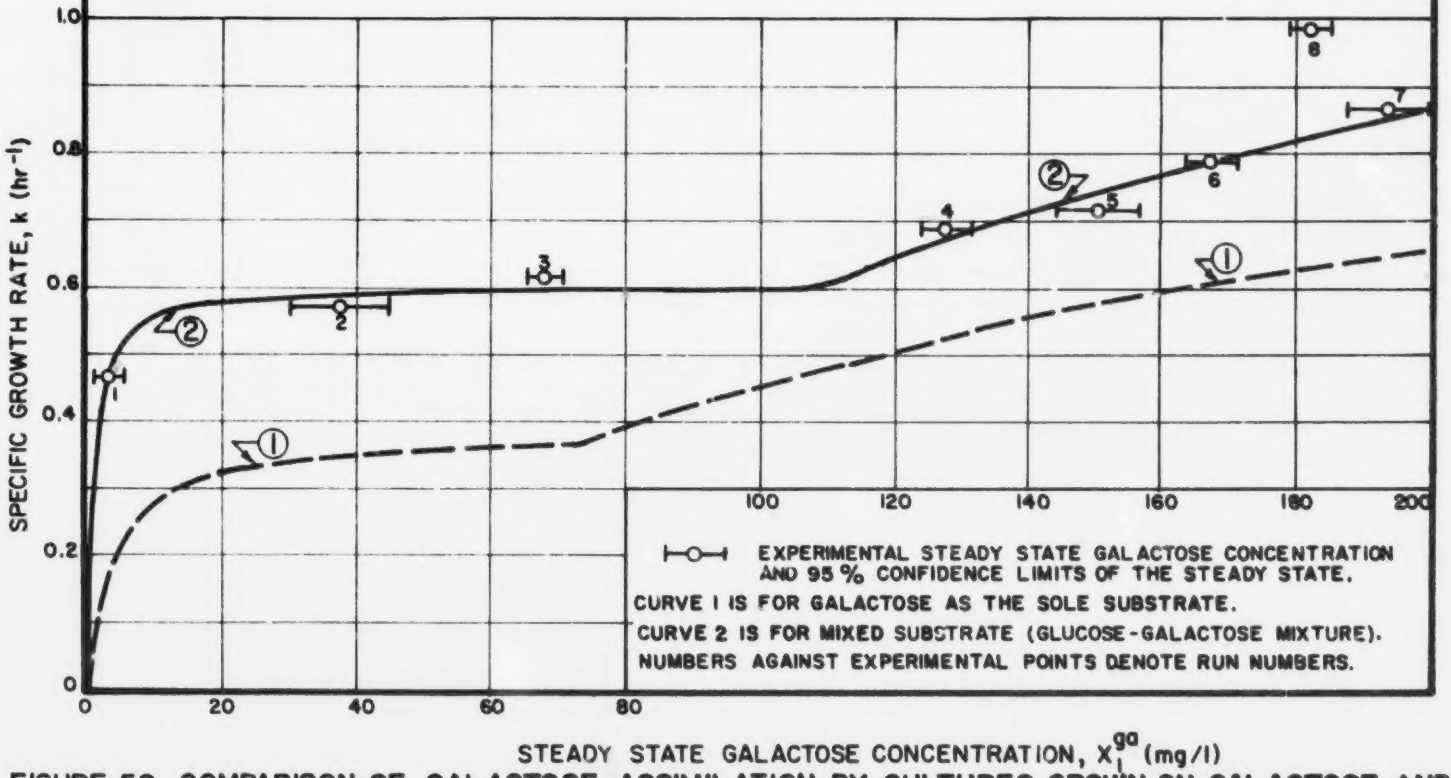
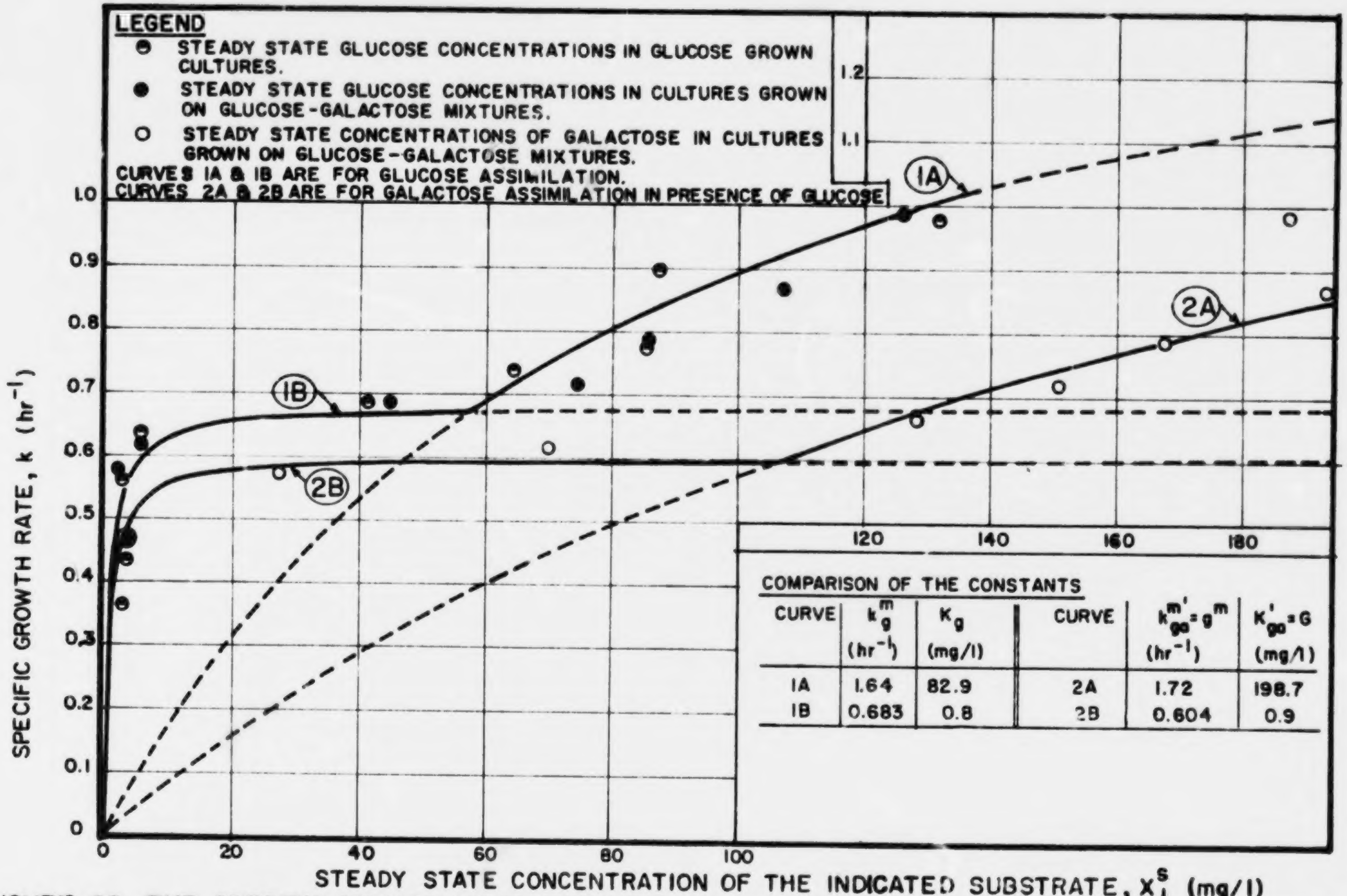


FIGURE 59. COMPARISON OF GALACTOSE ASSIMILATION BY CULTURES GROWN ON GALACTOSE AND ON GLUCOSE-GALACTOSE MIXTURES.



COMPARISON OF THE CONSTANTS

| CURVE | $k_g^m$ $(hr^{-1})$ | $K_g$ (mg/l) | CURVE | $k_{ga}^{m'} = g^m$ $(hr^{-1})$ | $K_{ga}' = G$ (mg/l) |
|---|---|---|---|---|---|
| 1A | 1.64 | 82.9 | 2A | 1.72 | 198.7 |
| 1B | 0.683 | 0.8 | 2B | 0.604 | 0.9 |

FIGURE 60. THE SPECIFIC GROWTH RATE AS FUNCTIONS OF GLUCOSE AND GALACTOSE CONCENTRATIONS FOR CULTURES GROWN ON MIXTURES OF GLUCOSE AND GALACTOSE.

were found to be concurrently assimilated at all growth rates. This observation is contrary to the contention of Gaudy and co-workers (211, 212), Stumm (216-218), and others (7,38) that a Class B sugar like galactose is spared in the presence of glucose and assimilated only in the second phase of substrate utilization. However, Baidya, *et al*. (134), Harte and Webb (135), Mateles, *et al*. (223), and Chian and Mateles (222) have shown that substrates, known to exhibit *diauxie* in batch cultures, could be concurrently utilized by continuous cultures at all dilution rates except those at near washout rates. Hernandez (219) also observed concurrent utilization of glucose and galactose in batch cultures.

*Secondly*, it was observed that, while galactose did not alter glucose uptake rates at any time, the presence of glucose definitely changed the kinetics of galactose assimilation very significantly. More importantly, galactose consumptions in the presence of glucose were substantially higher at all detention times than those possible when galactose was the only substrate. This particular finding was diametrically opposite to what had been suggested by other investigators (211,212, 216,218).

*Thirdly*, the function relating steady state galactose concentrations (in the presence of glucose) to specific growth rates indicated that the apparent $k^m$ on galactose increased (see table in Figure 59) for both the slow and the fast growing populations prevalent at high and low detention times, respectively. On the other hand, the saturation constant with galactose decreased for the slow growers to a value almost equaling the saturation constant with glucose (see table in Figure 60). However, for fast growers the saturation constant with galactose in the presence of glucose was greater than that with galactose as sole substrate.

Identification of the Sugar that Controlled Growth. An important question that should be answered before any other concerns the identification of the specific substrate which determined the specific growth rates. It had been established from the results of the galactose runs that galactose alone could not sustain specific growth rates of more than 0.3869/hr for the slow growers due to the limiting velocity of the galactose permease. Therefore, if galactose had been the growth determinant in the presence of glucose, the maximum specific growth rate of the slow growers (i.e., $k^m$ of Curve 2B in Figure 60) could not have been 0.604/hr as observed, since the rate determining step in galactose uptake cannot permit such a high growth rate for the slow growing species. From another viewpoint, it can be seen from Curve 1 of Figure 59 that, at a steady state concentration of 40 mg/ℓ of galactose, a specific growth rate of 0.35/hr was possible when this substrate controlled the growth rate as the sole substrate. However, if the specific growth rate is adjusted to 0.35/hr (by suitably adjusting the dilution rate) in the reactor with mixed sugar feed, then the steady state galactose concentration cannot rise above one mg/ℓ as given by Curve 2 of Figure 59. Had galactose been the growth limiting substrate, a specific growth rate of more than 0.05/hr (from Curve 1, Figure 59) could not have been possible at one mg/ℓ of galactose. Consequently, the high specific growth rate of 0.35/hr obtainable with the mixed feed system at one mg/ℓ steady state galactose could only be attributed to the presence of glucose, since a steady concentration of one mg/ℓ of the latter could sustain the above specific growth rate of 0.35/hr even when present as the only growth limiting substrate. It appears, therefore, that, with the mixed sugar substrate, glucose controlled the specific growth rate. The high growth yield (0.904) with mixed glucose-galactose and the fact that it was nearly equal to that (0.841) obtained with glucose as the sole substrate, provided further evidence that glucose was the growth determining substrate.

Kinetics of Galactose Uptake in the Presence of Glucose. Notwithstanding the aforementioned role of glucose, it is not clear *a priori* as to why the steady state galactose concentrations in the presence of glucose were so much lower than those predictable on the basis of the galactose run. For example, it had been established that, due to transport limitation, the extracellular galactose concentration could not be depressed below 15 mg/ℓ (as given by Curve 1 of Figure 59) at k = 0.3/hr. Yet, in the presence of glucose, the extracellular galactose (from Curve 2 of Figure 59) was reduced to two mg/ℓ. It remains to be resolved as to how greater quantities of galactose could be removed from the medium despite the physiological limitations of the galactose transport mechanism.

Figure 60, which compares the galactose uptake rates by cultures grown on galactose and galactose-glucose mixtures, proved to be a useful means in gaining a clearer insight into the transport capabilities of the cells in the galactose and the mixed sugar systems. Based on the assumption that the galactose uptake rate from the reactor is a direct function of galactose transportation rates by the permeases, it can be concluded from Figure 61 that the specific galactose transport rates (in terms of mass of galactose transported per unit biomass per unit time) by the cultures fed with mixed sugars were substantially lower than the

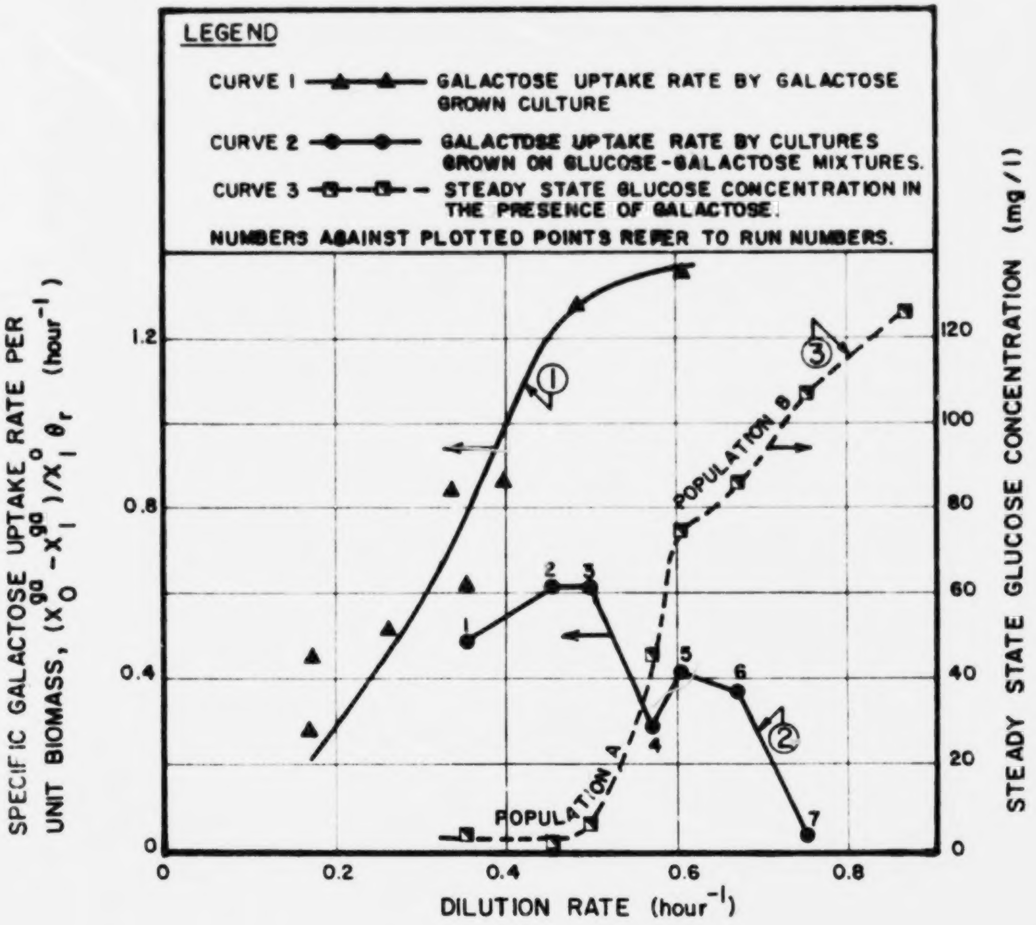


FIGURE 61. EFFECT OF GLUCOSE ON SPECIFIC GALACTOSE UPTAKE RATES.

rates of galactose transport by the galactose grown cultures at all dilution rates. The specific galactose transport rate by each glucose-galactose grown cell was lower at all detention times than one half the maximum specific galactose transport rate exhibited by a galactose grown cell. However, the high total consumptions of galactose in the presence of glucose resulted because the former substrate was used to satisfy the energy requirements of the high growth yield and culture density effected by the latter. The combined effect of the three factors, namely (a) use of galactose as the sole source of energy of maintenance, (b) high growth yield on glucose, and (c) high coefficient of energy of maintenance was to depress the steady state galactose concentrations to a level much below what could be effected by the galactose grown cultures having substantially lower growth yield and maintenance coefficients. The following example serves to illustrate this point.

Consider Run 1 with glucose-galactose mixture.

In Run 1:

$\theta_r = 2.84$ hours

$X_i^o = 85.8$ mg/ℓ from Table 15

$X_o^{ga} = 119.9$ mg/ℓ from Table 9

From Figure 55

$m'_{ga} = 0.485\ \text{hr}^{-1}$

Then total galactose consumption for satisfying the energy of maintenance $= X_i^o\ \theta_r\ m_{ga}$

Hence steady state galactose concentration,

$$X_i^{ga'} = X_o^{ga} - X_i^o\ \theta_r\ m'_{ga}$$

Substituting the values of parameters,

$$X_1^{ga'} = 1.9 \text{ mg}/\ell,$$

compared to the observed steady state concentration of 3.1 mg/ℓ. Therefore, at any detention time, the steady state galactose concentration was a function of the steady state organism concentration.

The hyperbolic nature of the function describing the relationship between the specific growth rate, k, and the steady state galactose concentration, $X_1^{ga'}$, in the reactor fed with mixed sugars can be explained by the following arguments: At any steady state, for which $X_1^o$ and therefore the concentration of each enzyme remains constant, the velocity of galactose transport by galactose permease, $V'_{ga}$, is given by

$$V'_{ga} = \frac{X_o^{ga} - X_1^{ga'}}{\theta_r} \tag{162}$$

but,

$$\frac{X_o^{ga} - X_1^{ga'}}{\theta_r} = mX_1^o \tag{163}$$

and

$$X_1^o = (X_o^g - X_1^g)/U'_g \tag{164}$$

also

$$X_1^g = f(k)$$

Therefore,

$$V'_{ga} = f'(k) \tag{165}$$

It is to be expected that $V'_{ga}$ and $X_1^{ga'}$ were related by a hyperbolic function of the Michaelis-Menten type, since permeation is an enzymatic reaction. Considering this and the relationship between $V'_{ga}$ and k, it appears that the steady state galactose concentration and the specific growth rate in mixed glucose-galactose systems should also be related through a hyperbolic function of the type described by Curves 2A and 2B of Figure 60.

In summary, with mixed sugar substrates it is the steady state glucose concentration, $X_1^g$, which determined the specific growth rate which in turn controlled the steady state galactose concentrations, $X_1^{ga'}$, or

$$k/_{\text{for growth on glucose + galactose}} = \frac{k_g^m X_1^g}{K_g + X_1^g} \qquad (166)$$

Since $X_1^{ga'}$ is a hyperbolic function of k as shown by Curves 2A and 2B of Figure 60

$$X_1^{ga'} = \frac{kK'_{ga}}{k^m_{ga} - k} \qquad (167)$$

where $k^{m'}_{ga}$ and $K'_{ga}$ are the uptake parameters for galactose and are related to the corresponding constants $V^{m'}_{ga}$ and $K'_{m_{ga}}$, respectively of the galactose permease reaction. Substituting Equation 166 into Equation 167

$$X_1^{ga'} = \frac{k_g^m K'_{ga} X_1^g}{k^{m'}_{ga} K_g + X_1^g (k^{m'}_{ga} - k^m_g)} \qquad (168)$$

Since $k^{m'}_{ga}$ and $K'_{ga}$ were not the maximum specific growth rate and the saturation constant for growth but were merely the uptake parameters for galactose consumption, these were replaced by $\underline{g^m}$ and G, respectively;

$\underline{g^m}$ and $\underline{G}$ are, respectively, related to the maximum velocity, $V^{m'}_{ga}$, and the Michaelis constant, $K'_{m_{ga}}$, of the rate limiting step for galactose uptake. Equation 168 then reduces to

$$X_i^{ga'} = \frac{k_g^m G X_i^g}{g^m K_g + X_i^g (g^m - k_g^m)} \qquad (168A)$$

In Equation 168A, $k_g^m$ and $K_g$ were the maximum specific growth rate and the saturation constant for the culture grown on glucose-galactose mixtures. The values of the constants of Equation 168A are noted in the table of Figure 60.

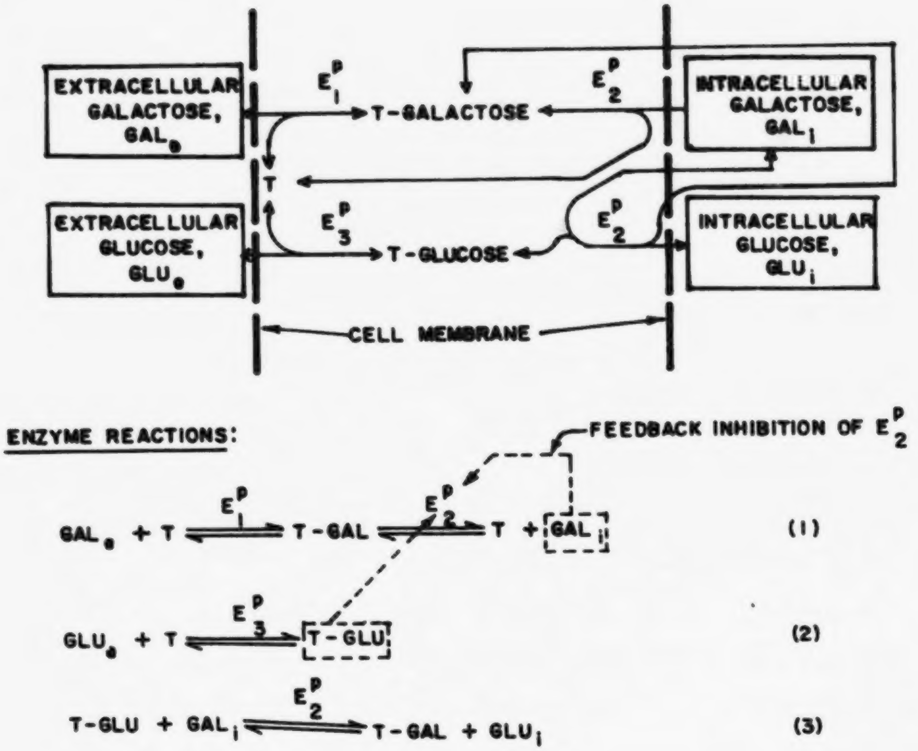
The significant conclusion to be drawn from the discussions of this section is that, when glucose and galactose were made available as the potential sources of carbon and energy, the cells of the heterogeneous cultures chose the former sugar as the growth limiting carbon source while relegating the latter to the role of a supplementary nutrient for providing the requirements of energy of maintenance. Use of the more efficient growth promoter, namely glucose, as the growth limiting substrate is logical, for only such a selection of substrate would allow a better expression of the maximum growth kinetic potential of the cells.

Probable Cellular Regulatory Mechanisms for Controlling the Kinetics of Galactose Uptake. Inspection of Curves 2 and 3 of Figure 61 showed that, in the presence of glucose, the galactose uptake rate--inhibited though it was--increased to a maximum corresponding to a glucose concentration of about six mg/ℓ and then decreased as the glucose concentration in the reactor increased. Horecker, Thomas, and Monod (20) have observed a similar variation of galactose transport rates by galactose permeases of *E. coli* with increasing concentrations of extracellular glucose. The parallelism of the experimental specific galactose uptake rates (Curve 2, Figure 61) with the galactose transport rates by galactose permeases as observed by Horecker, *et al*. (20) suggests that, in the case of the glucose and galactose grown cells, galactose uptake was controlled by the galactose permeases. This conclusion further substantiates the earlier contention that galactose permeation was the rate controlling step in galactose metabolism.

Comparison of Curves 1 and 2 of Figure 61 indicated that each glucose-galactose grown cell transported less galactose than a galactose grown cell and, as such, the formation of galactose permease and the three other galactose enzymes must have been repressed or their activities inhibited in the presence of glucose. It should be recognized that, for any detention time, the extracellular galactose concentration was lower in the presence of glucose than that in the absence of it; a lower external galactose concentration may have effected a lower degree of induction of the galactose enzymes. It has been postulated by Buttin (308,309) and Baidya, *et al*. (134) that, even at low extracellular concentrations of glucose, sufficient repressor catabolites are formed from glucose to repress synthesis of the galactose permease and the galactose enzymes. Thus, while extracellular galactose induced galactose enzymes commensurate with its concentration, glucose through its catabolites had the effect of repressing the formation of these enzymes. According to Magasanik (163), a steady state can be presumably reached where induction and repression set the level of the galactose permease and the galactose enzymes.

In addition to the mechanisms of controlled induction and repression, many organisms have another mechanism for controlling the activities of the galactose permease and the galactose enzymes. According to the Permease Model of Horecker, *et al*. (20) (see Figure 62), both glucose and galactose can form complexes with the *transporter* substance, T, suggested by Kepes (277). Furthermore, transporter bound glucose is capable of undergoing an exchange reaction with the transported intracellular galactose to liberate free glucose inside the cell. From Reaction 3 of Figure 62, it is apparent that, as the concentration of extracellular glucose was increased (as by increasing the dilution rate), liberation of internal glucose and T-galactose was accelerated. Accelerated production of T-galactose would increasingly inhibit transport of extracellular galactose. It is evident that the galactose permease $E_a^p$ is inhibited by T-glucose and galactose; also, that the latter substrate is a product of the reaction (Reaction 1) catalyzed by $E_a^p$ itself. The inhibition of the galactose permease can thus be classified as a *negative feedback inhibition*. The Michaelis-Menten type of plot of Figure 63 indicated that the inhibition of galactose permease involved lowering of the maximum specific uptake rate. The saturation constant for galactose uptake did not increase but decreased (compare $K_{ga}'$ with $K_{ga}$ in Figure 59) in the presence of glucose. These observations indicated that glucose brings about a *non-competitive feedback inhibition* of the galactose permease. The inhibition of galactose permease is of importance in controlling galactose uptake rate in organisms constitutive in galactose permease.

Curve 2 of Figure 61 indicated that the galactose transport rates


EXTRACELLULAR GALACTOSE, GAL$_e$
EXTRACELLULAR GLUCOSE, GLU$_e$
INTRACELLULAR GALACTOSE, GAL$_i$
INTRACELLULAR GLUCOSE, GLU$_i$
$E_1^P$
$E_2^P$
$E_3^P$
$E_2^P$
T-GALACTOSE
T
T-GLUCOSE
CELL MEMBRANE
FEEDBACK INHIBITION OF $E_2^P$

ENZYME REACTIONS:

$$GAL_e + T \overset{E_1^P}{\rightleftharpoons} T\text{-}GAL \overset{E_2^P}{\rightleftharpoons} T + \boxed{GAL_i} \qquad (1)$$

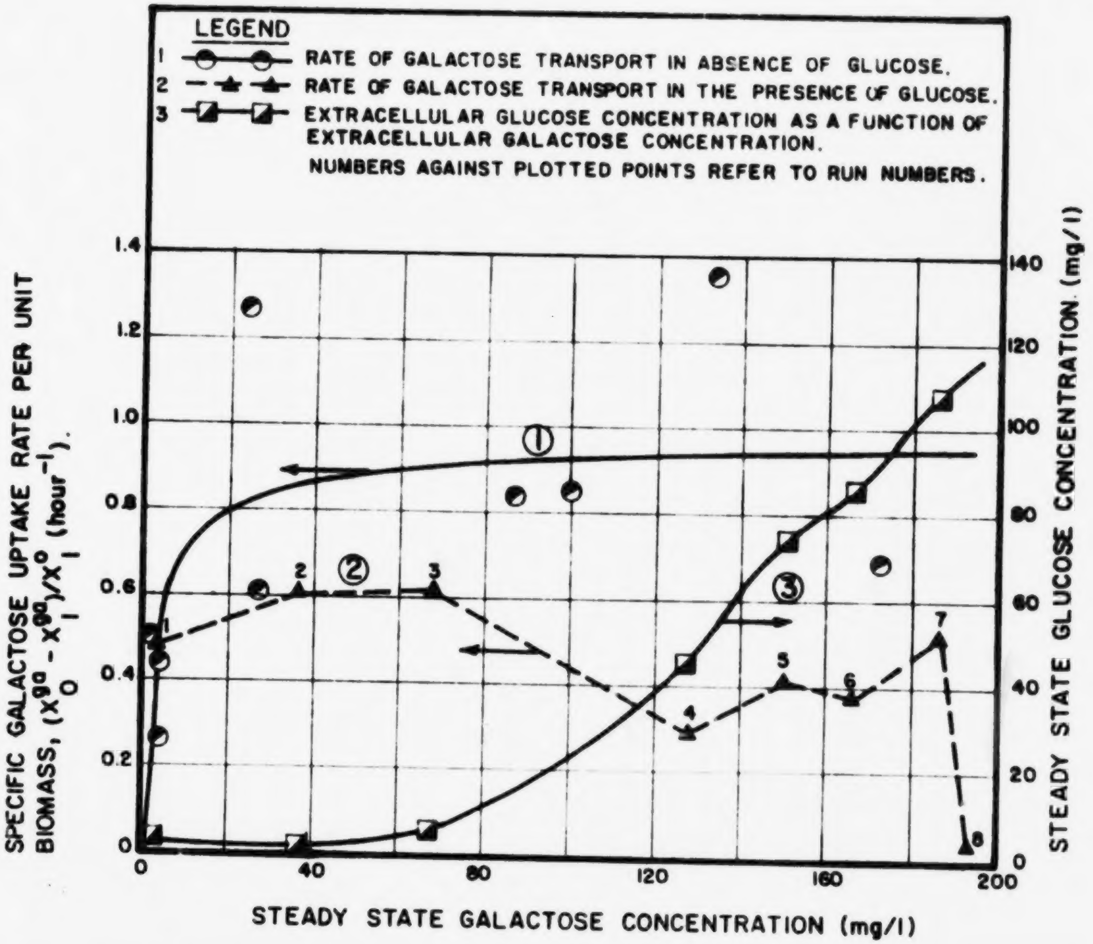
$$GLU_e + T \overset{E_3^P}{\rightleftharpoons} \boxed{T\text{-}GLU} \qquad (2)$$

$$T\text{-}GLU + GAL_i \overset{E_2^P}{\rightleftharpoons} T\text{-}GAL + GLU_i \qquad (3)$$

NOTES:

1. $E_1^P$ AND $E_2^P$ ARE ENZYMES OF THE GALACTOSE PERMEASE SYSTEM.
2. $E_3^P$ IS AN ENZYME OF THE GLUCOSE PERMEASE SYSTEM.
3. T IS A TRANSPORTER SUBSTANCE
4. $E_2^P$ FAVORS FORMATION OF T-GAL AND GLU$_i$ TO "T" AND GAL$_i$
5. T-GAL = T-GALACTOSE
6. T-GLUCOSE = T-GLU

FIGURE 62. SCHEMATIC REPRESENTATION OF GALACTOSE TRANSPORT IN THE PRESENCE OF GLUCOSE.
(ADAPTED FROM THE HORECKER-JACOB-MONOD MODEL (20)(21)).



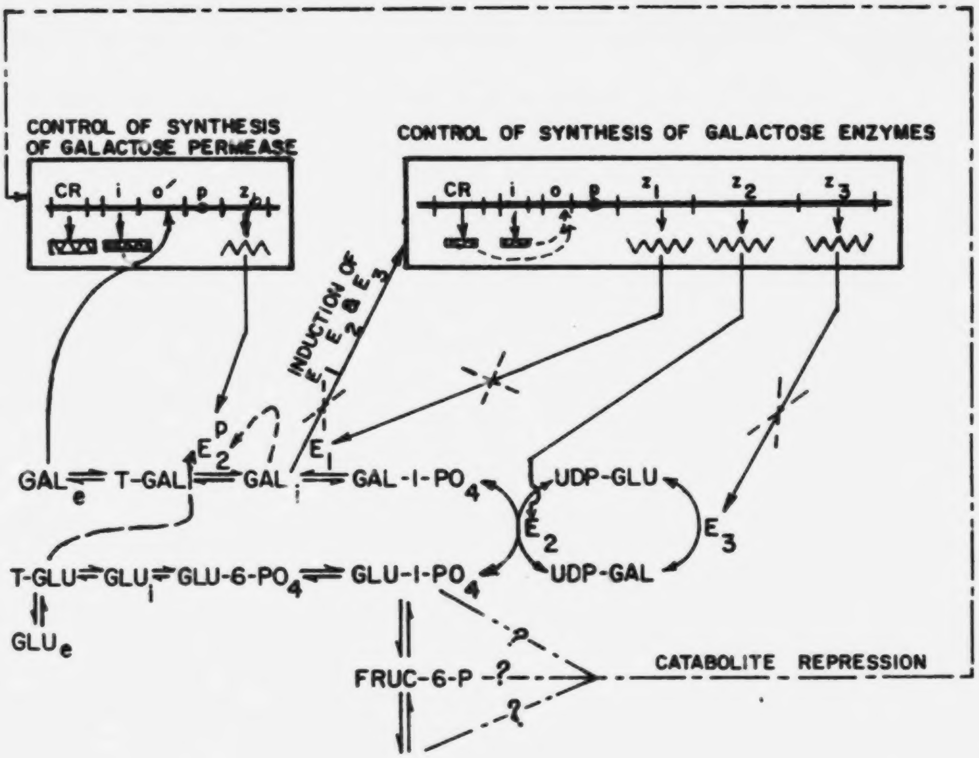
FIGUCE 63. INHIBITION OF SPECIFIC GALACTOSE UPTAKE RATES IN THE PRESENCE OF GLUCOSE.

by each cell steadily decreased with increase in dilution rate in the presence of glucose, which was the reverse of what happened in the absence of glucose. This fact indicated that either the cellular content of the galactose enzyme or the activities thereof decreased despite higher extracellular concentration of the galactose (the inducer) at higher dilution rates. This is explained by the fact that the high glucose concentration at high dilution rates brought about increased inhibition of the constitutive galactose permease, as mentioned in the preceding paragraph. The resulting intracellular galactose concentration after inhibition cannot effect as much induction of the galactose enzymes as it would in the presence of lower concentrations of glucose, because the latter substrate increases the threshold intracellular concentration of the former which is necessary for stimulation of the *galactose operon* (185,272,405). Thus, at certain high steady state concentrations of glucose, the intracellular galactose concentration (which also depends on its extracellular concentration according to Reaction 1 of Figure 62) may be such that it falls below the threshold concentration required for induction of the galactose enzymes. The result will be that no galactose can be metabolized if the ratio of galactose and glucose concentration falls below a certain value. Thus, when $X_0^{ga}$ is below $X_1^{ga'}$, computed from Equation 168 for a given value of $X_1^{g}$ and $\theta_r$, galactose will pass through the reactor unmetabolized.

For strains of organisms with inducible galactose permeases, higher glucose concentrations mean higher concentration of *repressor catabolites* for the galactose *operon*. The degree of *catabolite repression* of the galactose enzymes increases with increasing concentration of the repressor catabolite (185) resulting from increasing the glucose concentration. The net effect is increasingly repressed formation of the galactose permease and the galactose enzymes as glucose concentration is increased.

Figure 64 is a schematic representation of the various mechanisms which may be responsible for regulating the galactose enzymes and galactose uptakes. Biosynthesis of the inducible galactose permease, $E_a^p$, is controlled by the same *regulator gene*, *i*, as for the three galactose enzymes $E_1$, $E_2$, and $E_3$, but the *regulator gene* acts upon a distinct *operator* site, $o'$, for initiation of permease synthesis (174,273,308,309). Some catabolite (the particular catabolite is not known) of glucose serves as the *repressor catabolite* and combines with the *aporepressors* of the *CR gene* to control or block transcription of the *structural genes*, $z_p$, $z_1$, $z_2$, and $z_3$, controlling the synthesis of the galactose permease and the galactose enzymes.

The lowering of the saturation constant for galactose assimilation at low extracellular concentrations of glucose merely pointed to a reversal of the inhibitory role of glucose. The observation that $K'_{ga}$ was less than $K_{ga}$ may be interpreted to mean increased affinity of the cells for galactose and increased consumption of it at very low extracellular concentrations of this substrate. The variation of *K* for galactose may be attributed to the variation of *K* for galactose permease observed in *E. coli* by Rotman and Radojkovic (274). Some authors also believe that increased transport of galactose at low galactose concentration may be due to the fact that other permease systems, including glucose permeases, may also mediate galactose transport (14,24,406).


CONTROL OF SYNTHESIS OF GALACTOSE PERMEASE
CR
i
o'
p
z
CONTROL OF SYNTHESIS OF GALACTOSE ENZYMES
CR
i
o
p
z1
z2
z3
INDUCTION OF E1 E2 & E3
E2p
E1
GALe
T-GAL
GALi
GAL-1-PO4
UDP-GLU
E2
E3
UDP-GAL
T-GLU
GLUi
GLU-6-PO4
GLU-1-PO4
GLUe
FRUC-6-P
?
?
?
CATABOLITE REPRESSION

LEGEND:

$GLU_e$ - EXTRACELLULAR GLUCOSE

$GAL_e$ - EXTRACELLULAR GALACTOSE

$GAL_i$ - INTRACELLULAR GALACTOSE

$GLU_i$ - INTRACELLULAR GLUCOSE

▨ APOREPRESSOR

CR - REPRESSOR GENE (AFTER LOOMIS & MAGASANIK (135))

$E_1$ $E_2$ & $E_3$ - GALACTOSE ENZYMES (SEE FIGURE 4)

$E_2^p$ - GALACTOSE PERMEASE

CR i o p z GENES CONTROLLING ENZYME SYNTHESIS (SEE FIGURE 5 FOR DETAILED EXPLANATION)

⟶ INDUCTION (POSITIVE FEEDBACK)

>-< PREVENTION OF INDUCTION AND SYNTHESIS OF ENZYMES

∿∿ mRNA TAKING PART IN APOENZYME SYNTHESIS

- - - → FEEDBACK INHIBITION

— - → NON-COMPETITIVE INHIBITION

FIGURE 64. SCHEMATIC REPRESENTATION OF CELLULAR MECHANISMS DEEMED RESPONSIBLE FOR REGULATION OF GALACTOSE UPTAKE IN THE PRESENCE OF GLUCOSE.

Galactose intake through multiple permease systems would be tantamount to a lowering of the saturation constant for galactose. Whatever the cause of the stimulated galactose utilization at lower concentrations of glucose, it appears that the lower $K'_{ga}$--which was almost equal to $K_g$ (see table in Figure 60)-- aided the organisms in sustaining the higher growth rates allowable by the prevailing glucose concentration.

Dehydrogenase Activities in Cultures Grown on Mixtures of Glucose and Galactose. In an earlier section it was reported that dehydrogenase activities of cultures fed with mixed glucose-galactose were substantially lower than those of the glucose or the galactose grown cultures. Lines 1 and 3 of Figure 65 represent the correlations between the active solids concentrations (noted in Table 15) and the dehydrogenase activities, measured, respectively, at light paths of one and ten centimeters, of the reactor cultures in the mixed substrate systems. Comparisons of correlation Lines 1 and 3 with the calibration Lines 2 and 4, respectively, established with single substrates (see Figures 29 and 30), indicated that cells grown on mixed sugars appeared to have less than one half the dehydrogenase activities of those grown on single sugars. It should also be noted that the cultures fed with mixed sugars metabolized more substrate per unit biomass compared to those grown on single sugars. For example, it can be calculated from the growth constants that, at a specific growth rate of 0.62/hr, cells grown on glucose, galactose, and on mixtures of the two metabolized 1.87, 1.80, and 2.08 milligrams of the respective substrates per milligram of biomass. If the quantity of substrate processed per unit biomass determines the quantity of dehydrogenase present, then glucose grown cells should exhibit slightly higher

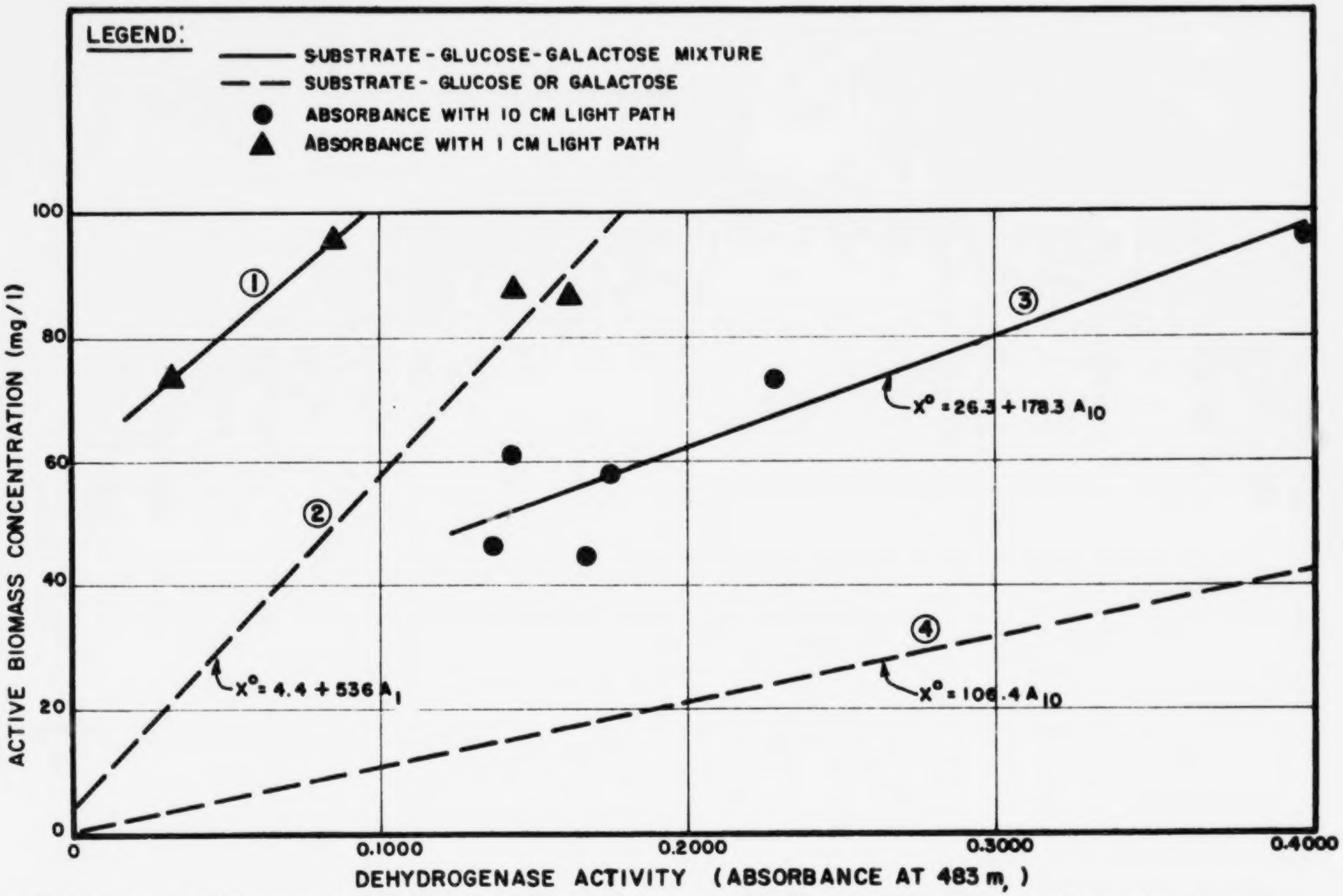


FIGURE 65. COMPARISON OF DEHYDROGENASE ACTIVITIES OF BIOMASS GROWN ON SINGLE AND MIXED SUGARS.

dehydrogenase activity. However, if one considers that galactose utilization involves an additional dehydrogenase bound to the UDP-epimerase (see Figure 4), then one might expect the dehydrogenase activities of the glucose and the galactose grown cells to be nearly equal, which is what was concluded from the calibration lines of Figure 29.

However, it is difficult to explain why unit biomass grown on the mixed sugars contained apparently less than one half of the dehydrogenase concentration present in cells grown on single sugars and yet metabolized more substrate. It may be postulated that the metabolic routes used for the metabolism of mixed sugars involved lesser numbers of dehydrogenases. In view of the known pathways of glucose and galactose metabolism (see Figure 4), it is difficult to accept such a hypothesis. It appears probable, however, that the organisms manage to metabolize more substrate with smaller quantities of dehydrogenases by increasing the turnover rates of these enzymes several fold. Such a scheme of operation would be preferable as this leads to the conservation of the carbon and energy sources needed, if increased concentrations of dehydrogenases were to be maintained. The conserved substrates could conceivably be used for synthesis of the galactose permease and the galactose enzymes. Whatever may be a realistic explanation for the smaller concentrations of dehydrogenases in glucose-galactose grown cultures, there seemed to be little doubt about the decreased dehydrogenase activities per unit quantity of biomass for such a heterogeneous culture. An important conclusion related to practical application of the dehydrogenase test is that any correlation established between dehydrogenase activity and biomass concentration on a particular substrate cannot be used for another substrate system.

## Dynamics of Heterogeneous Microbial Populations in Continuous Culture

The results of the continuous culture experiments with glucose, galactose, and glucose-galactose mixtures indicated that the heterogeneous populations at the low and high detention times were very different from each other in terms of their growth kinetic properties.* The occurrence of microbial populations of varying growth kinetic properties at different detention times is attributable to the environmental changes imposed by variation of the dilution rates. Generally, for both sugars the dominant species at detention times higher than three hours were characterized by lower maximum specific growth rates, $k^m$, and saturation constants, K. Any species with high $\underline{k^m}$ and $\underline{K}$ (hereafter referred to as the fast growers) was at a disadvantage since the dominant species at high detention times (referred to as the slow growers) could grow more efficiently at the low substrate concentrations associated with the higher detention times. However, as the detention time was decreased (i.e., as the dilution rate was increased), the slow growers gradually lost their growth efficiency as substrate concentration increased. The population density of the slow growers gradually diminished relative to those of the fast growers. Also, each stepped increase in the dilution rate effected the gradual elimination by washout of those slow growers whose maximum specific growth rates were exceeded by the increased dilution rate. It is not difficult to envision, therefore, that the fast growers, which might have been present in very small cell concentrations at the high detention times, became increasingly dominant with the increase in dilution rates. Apparently the slow growing species lost their dominance almost completely to the fast growers when the substrate concentration reached a critically high level (approximately 60 ppm for glucose and 70 ppm for galactose). This conclusion is in agreement with that made in Chapter IV from purely theoretical considerations.

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*The maximum specific growth rate constant, $k^m$, and saturation constant, K, are referred to as the growth kinetic properties of a microbial population.

The phenomenon described herein can be best illustrated with reference to the actual data. For example, if the glucose reactor were operated at $\theta_r = 2.6$ hrs (see Figure 45), all species capable of achieving a mean generation time, $\bar{\tau}$, of 1.4 hours (and $k = 0.5$/hr) survived. However, all species are not endowed with similar growth capabilities. Novick (74) and Novick and Aaron (73) pointed out that every species exhibits a minimum limit of generation time or a maximum limit for $\underline{k^m}$. The data from the glucose runs indicated that the two groups of species with the following limits of generation time and maximum specific growth rate were present:

Population A, slow growers:

$$\bar{\tau}_{min} = 0.95 \text{ hr}, \quad k^m = 0.73/\text{hr}, \text{ and } K = 1.72 \text{ mg}/\ell$$

Population B, fast growers:

$$\bar{\tau}_{min} = 0.49 \text{ hr}, \quad k^m = 1.41/\text{hr}, \text{ and } K = 59.9 \text{ mg}/\ell$$

It may be noted that the larger $\underline{k^m}$ was associated with the larger $\underline{K}$, and the lower $\underline{k^m}$ was associated with the lower $\underline{K}$. These observations were consistent with the conclusion made by Jannasch (225,226,239) that usually a species capable of growing at high specific growth rate exhibits poorer affinity (i.e., a high K value) towards the substrate at low concentrations.

Returning now to reactor operation at $\theta_r$ = 2.6 hrs, and also considering the specific growth rate functions (see Figure 45) defined by the growth rate constants of slow and fast growers, it becomes apparent that the slow growers tend to lower reactor glucose concentration to four mg/ℓ. The fast growers who are inefficient at this detention time can only lower the glucose concentration to 33 mg/ℓ. Obviously, Population A (slow growers) predominated at the detention time of 2.6 hours by virtue of the fact that their glucose consumption and growth yield were at least eight times as much as could be effected by Population B (fast growers). The inefficiency of the fast growers at the detention time of 2.6 hours was largely due to the larger saturation constant of the member species. Population B may have survived only at very low population densities because of the adverse selection pressure* determined largely by the difference in the values of the saturation constants ($K_B$-$K_A$).

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*In a chemostat type reactor there are two important factors which determine the survival and the population density of a species relative to those of the other species. If a reactor is operated at dilution rate, D, such that (D-$k_j^m$) for species j becomes positive, then it is obvious that species j would be washed out. The higher the value of the intensive parameter (D-$k_j^m$), the quicker will be the washout of species j. The parameter (D-$k_j^m$), the value of which determines whether species j will be selected against or for survival at dilution rate, D, may be termed as the selection pressure on species j.

A species A with lower saturation constant, K, consumes more substrate thereby yielding higher population density, especially at the lower dilution rates. Species B with a higher K do not have this advantage and may survive only at comparatively lower densities. Species B cannot attain as high a cell density as that of Species A because of the difference between the saturation constants ($K_B$-$K_A$), which is also an intensive parameter. The selection pressure on Species B is ($K_B$-$K_A$), because as the value is increased the cell density of this species relative to that of Species A would decrease, i.e., Species B would be increasingly selected against.

If reactor operation at $\theta_r$ = 1.28 hours with glucose is considered (see Figure 45), it is evident that Population A could not have survived as the member species would not be capable of adjusting to a generation time of 0.77 hour (or a $k^m$ of 0.9/hr) which is much below the minimum generation time, $\bar{\tau}_{min}$ of 0.95 hour, of these species. Consequently, only the constituent species of Population B survived at this detention time, while those of Population A were washed out. The effective selection pressure against Population A was therefore the difference between the dilution rate employed and the maximum specific growth rate of this population.

The essence of the preceding discussion is that the species composition of mixed continuous cultures does not remain the same at all dilution rates. Heterogeneous microbial populations in chemostat type reactors are subjected to selection pressures. The species achieving the fastest growth rate and higher population density under a given set of environmental conditions approaches a steady state, while the competing species are gradually displaced (237,407). For any species there is a critical dilution rate at which the species will be completely washed out. While lower dilution rates may allow the existence of many species, only fewer fast growing species can survive at higher dilution rates. Theoretical considerations as well as the varying nature of the growth kinetic constants led to the conclusion that the physiologic capability and the species composition of the culture were a dynamic function of the detention time. Although no taxonomic studies of the cultures were undertaken, a number of references in support of the phenomenon of selection in chemostat type reactors exist (58,61,74,76,77,88,222,225,226, 240,252,253,267,268). Jannasch (226), Collard and Gossling (268), Chain and Mateles (222), Mateles (224), and Dias and Bhat (266) reported that, in mixed continuous cultures, *E. coli*, *S. faecalis*, *Bacteroides*, *S. albicans*, *S. albus*, *Achromobacter*, etc. dominated at higher detention times whereas species belonging to the genus *Pseudomonas* were dominant at low detention times. The selective property of the dilution rates in chemostat type reactors has been used by pure culturists (238,252) as well as sanitary engineers (96,99) to either isolate various bacterial strains or for the proliferation of desired dominants (such as acid or methane formers (96) or nitrifiers (99)), to effect the required biochemical conversions.

Appreciation of the phenomenon of population dynamics serves to illuminate some of the operational peculiarities of continuous cultures not completely understood at the present time. It has been recognized by many that oscillatory steady states are inevitable with mixed cultures (222,253,398,401). Some investigators have doubted the attainability of steady states with mixed cultures due to their very unstable nature, whereas others have suggested mathematical means for optimizing the use of the oscillatory data (400,401). However, in light of the preceding discussion on variability of steady states, stability of steady states can be considered a direct function of dilution rate and an inverse function of the heterogeneity of the culture. It may be pointed out in this context that the existence of steady states, like beauty, is in the eyes of the beholder. However, fluctuations of steady state concentrations may be expected to be more at lower dilution rates because of the increased degree of heterogeneity. If slime growth and variability of flow rates are not controlled at this range of dilution rate, then it may become impossible to attain steady states under these conditions.

As far as it could be determined from the literature, it has been the usual practice to characterize all the populations at different experimental detention times by single values of $\underline{k}^m$ and $\underline{K}$. This technique is tantamount to assuming that the species composition of the culture does not change with change of dilution rate. Curves marked 1 in Figures 45 and 49 were obtained by this procedure. The fact that these curves afforded poor fits to the data has already been stressed. A number of investigators have fitted the continuous culture data in the above manner and have variously attributed the poor fit to "imperfect" mix, inadequacy of the Monod growth rate function, or to some unknown factors (58,59,60). Slime growths and imperfect mixing could not have caused the poor fit of data to Curve 1 of Figures 45 and 49 as the slime formation was controlled to insignificant levels, and the data of Figure 36 testify to the attainment of complete or "perfect" mix. It is contended that poor fits of data by "average" $\underline{k}^m$ and $\underline{K}$ resulted not because of the inadequacy of the Monod Equation but because the "average" constants characterized neither the slow growers nor the fast growers. Based on the preceding data and associated discussions, it is apparent that the use of multiple curves, as in Figures 45 and 49, is a more realistic approach to analyzing the kinetic characteristics of cultures.

Based on the "average" growth rate curves (Curve 1) of Figures 45 and 49, complete washout of all organisms is predicted at detention times below 1.4 and 2.3 hours for the glucose and the galactose cultures, respectively. However, no washouts were obtained at several detention times below the critical values mentioned above. Such failure to achieve washouts had also been reported and unexplained by various investigators (64,65,58,134,135). This phenomenon of no washout can be explained in light of the dynamics of population composition in continuous reactors. As the maximum specific growth rate of the dominants at low detention times far exceeded the $\underline{k}^m$ of the "average" Curve 1 (see tables in Figures 45 and 49), no washout could be legitimately anticipated.

The growth kinetic characteristics of the slow and the fast growers are summarized in Table 17 for the two sugar substrates. The critical dilution rate at which the slow growers ceased to be the dominant population can be determined from Equation 122 of Chapter IV. Thus, the theoretical detention times at which the population change overs (i.e., from Population A to Population B) are presumed to have taken place were 1.70 and 2.94 hours, respectively, for glucose and galactose substrates. The corresponding critical substrate concentrations, as given by Equation 124 of Chapter IV, were 60.3 mg/ℓ of glucose and 72.3 mg/ℓ of galactose. The critical detention time and the substrate concentrations are also noted in Table 17.

Although rigorous taxonomic studies for identification of species were outside the scope of this investigation, it was decided to examine and record if there occurred any obvious changes in the overall gross properties of cultures growing at high and low detention times. Some standard methods of bacteriological examinations (327), as noted in Table 18, were chosen for this purpose. Pigment-forming properties of the culture, used by Cassell, et al. (270) for detecting changes in population composition, were studied by determining the absorption peaks

Table 17. Comparison of Growth Kinetic Constants of Dominant Microbial Populations at High and Low Detention Times

| Substrate | Kinetic Constants of Dominant Populations | | | | Critical Points of Population Change Over | | |
|---|---|---|---|---|---|---|---|
| | Population A slow growers | | Population B fast growers | | Dilution Rate | Detention Time | Substrate Conc. |
| | $k_A^m$ ($hr^{-1}$) | $K_A$ (mg/$\ell$) | $k_B^m$ ($hr^{-1}$) | $K_B$ (mg/$\ell$) | D ($hr^{-1}$) | $\theta_r$ (hr) | $X_1^s$ (mg/$\ell$) |
| Glucose | 0.7280 | 1.72 | 1.4112 | 59.9 | 0.588 | 1.70 | 60.3 |
| Galactose | 0.3869 | 3.9 | 1.1284 | 149.9 | 0.340 | 2.94 | 72.3 |

of the alcohol extracts prepared from a reactor sample drawn at several test detention times. From Table 18 it is apparent that the dominant culture which thrived on galactose at detention times of six and two hours exhibited distinctly different Gram staining and pigment forming properties. The coliform count was high at high detention time, an observation which was in agreement with that of Chian and Mateles (222) and Dias and Bhat (266). Protozoan concentration was zero or very small at low detention times probably because the low bacterial concentration could not support a predator population in any significant number.

The cultures grown on glucose at detention times of four and 1.75 hours did not exhibit as much difference in pigment forming properties and protozoan population as observed with the galactose cultures, although the Gram staining properties and coliform count were considerably dissimilar. However, as shown in Table 17, cultures at four and 1.75 hours detention time should have some similarities since both detention times are higher than the critical detention time (1.70 hours) of pro-

Table 18. Some Properties of the Dominant Cultures A and B at High and Low Detention Times of Continuous Flow Reactor

| Substrate | Appearance of Colonies on Glucose Agar Plate | Predators | Gram Stain | Coliform* Count (no./ℓ) | Wavelength of Absorption Peak of Alcohol Extract ($m\mu$) |
|---|---|---|---|---|---|
| Glucose ($\theta_r$ = 4 hrs) | 1. Predominantly tiny opaque colonies<br>2. Some large opaque colonies | Free swimming protozoa | 1. Predominantly short (4 $\mu$) gram negative rods<br>2. Some gram positive short rods<br>3. Some gram positive cocci | $8 \times 10^5$ | 300 |
| Glucose ($\theta_r$ = 1.75 hrs) | 1. Predominantly small opaque colonies<br>2. Some large opaque colonies | Some protozoa | 1. Higher percentage of long (8 $\mu$) and slender gram positive rods<br>2. Short (4 $\mu$) crescent shaped gram negative rods<br>3. Some gram negative cocci | $2 \times 10^5$ | 300 |

Table 18. Some Properties of the Dominant Cultures A and B at High and Low Detention Times of Continuous Flow Reactor (Concluded)

| Substrate | Appearance of Colonies of Glucose Agar Plate | Predators | Gram Stain | Coliform Count* (no./ℓ) | Wavelength of Absorption Peak of Alcohol Extract (mμ) |
|---|---|---|---|---|---|
| Galactose ($\theta_r$ = 6 hrs) | Tiny white opaque colonies | Free swimming protozoa | 1. Short gram negative rods | $9 \times 10^4$ | 320 |
| Galactose ($\theta_r$ = 2 hrs) | Large white opaque colonies | No protozoa observed | 1. Predominantly gram positive rods<br>2. Some gram positive cocci<br>3. Some gram negative rods | | 294 |

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*membrane filter technique

nounced population change over. A general conclusion which can be made from Table 18 is that, for both substrates, the population at high detention times contained higher concentrations of Gram negative rods, coliform bacteria, and predators. In this respect, the data were in good agreement with those of Chian and Mateles (222) and Dias and Bhat (266).

The foregoing discussion was in reference to two distinctly different and easily identifiable populations (namely, Population A and Population B). However, it was not implied to mean that the species diversity and the relative population density of different species remained identical at all detention times above and below the "critical point of population change over" (see Table 17) which heralded an abrupt change in the population composition. Theoretically, any change in detention time is necessarily associated with a change in the relative population densities of the different species and species diversity. However, in some ranges of detention times, the changes of population composition may not be reflected as significant changes in the growth kinetic capabilities of the total population. The "critical points of population change over" of Table 17 should be regarded as describing one of the transitional environments separating the environments for the slow and the fast growers. In the system studied, a very marked change of microbial flora occurred at glucose and galactose concentrations of 60 mg/ℓ and 72 mg/ℓ, respectively.

Shifts in population composition were also evident in the continuous culture runs with glucose-galactose mixtures. It is interesting to note from Figure 57 that the steady state glucose concentration which separated the predominance of slow and fast growers was the same for glucose and glucose-galactose grown cultures.

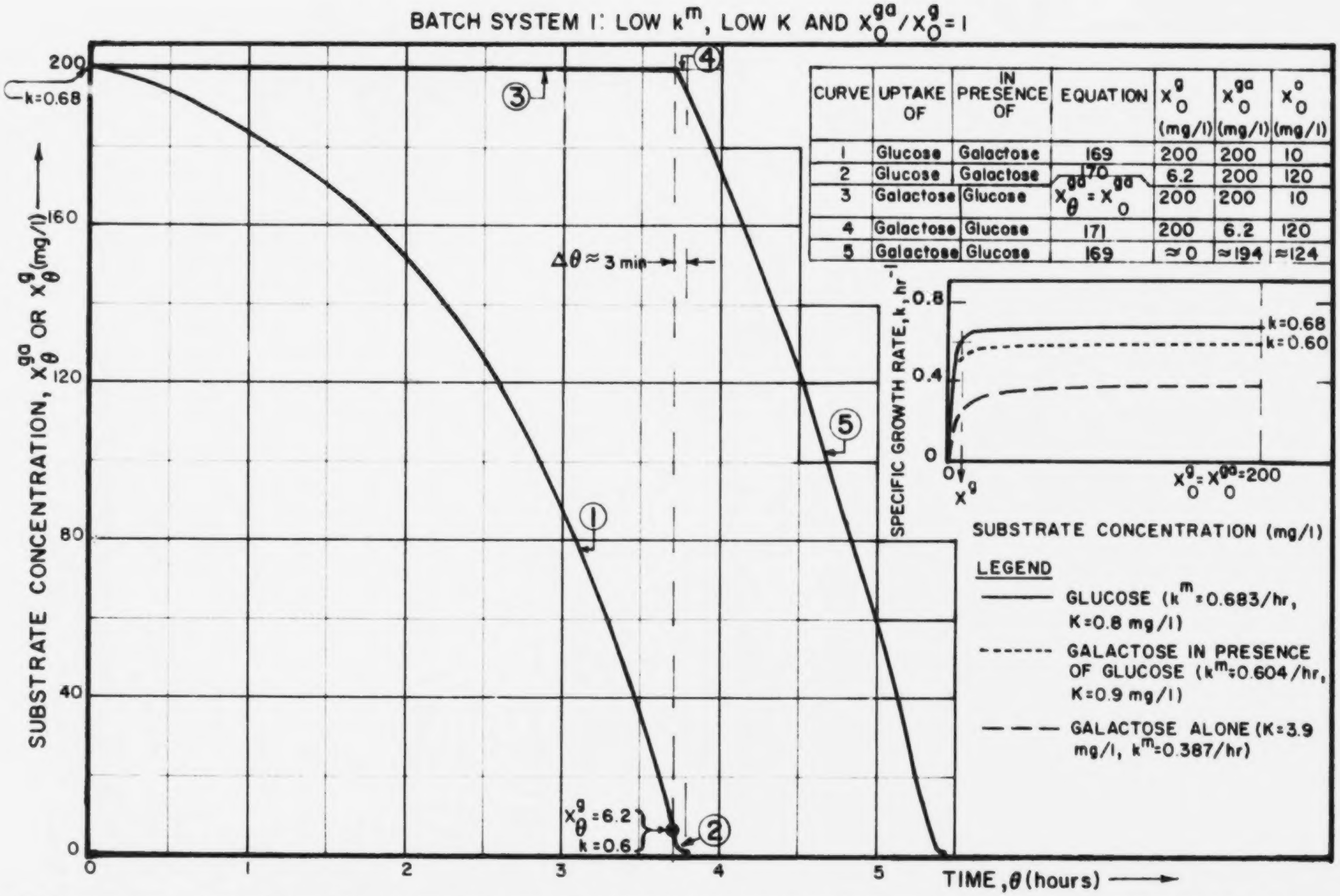
## Role of Environmental Factors in Determining the Kinetics of Uptake of Interacting Substrates

Despite the reduced uptake of galactose by each cell in the presence of glucose due to feedback type inhibition of the galactose permease, glucose could not prevent cellular intake of galactose. Both sugars were concurrently utilized. Concurrent utilization of diauxic substrates in continuous flow reactors has also been observed by others (134,135,222,223). The results of this research were also in agreement with those of Hernandez (219) who observed concurrent utilization of glucose and galactose in batch cultures. Concurrent assimilation of diauxic substrates (glucose and sorbitol) were also observed by Gaudy, et al. (213). There are, however, several other reports in which glucose was reported to have, almost completely, suppressed the utilization of secondary substrates like galactose, thus giving rise to sequential uptake of substrates (216-218,211-214). No explanation has been presented by the researchers concerned to account for the contradictory results.

As will be revealed shortly, several environmental factors play important roles in determining whether the uptake of the two interacting substrates should be concurrent or phasic. It should be reemphasized that the choice between the two modes of uptake of interacting substrates should be such that it will enable the microorganisms to grow at the maximum possible rate and with the maximum possible yield of biomass. In the following discussions, a few important biological systems are subjected to theoretical analyses in terms of the possible nature of the kinetics of substrate uptake. Glucose and galactose were selected as the interacting substrates. The objective was the development of a generalized hypothesis which is capable of accounting for the various contradictory results on assimilation of interacting substrates.

Interacting Substrates in Batch Reactors. Since there is no opportunity for population change overs with the progressive change of growth rate during batch utilization of substrates, complex growth curves, such as those of Figures 45, 49, and 60, are not needed to describe the specific growth rates of batch cultures. The specific growth rates at all times will be defined by a single $\underline{k}^m$ and a single $\underline{K}$, since the physiological character of the microbial population remains unaltered in batch.

Figure 66 shows the theoretical curves for batch utilization of glucose and galactose by a slow-growing heterogeneous population, similar to Type A of Figures 45, 49, and 60 and characterized by low $\underline{k}^m$ and $\underline{K}$ when the initial concentrations of the two substrates bear a ratio of 1:1. Curves 1B and 2B of Figure 60 describe the specific growth rates of this microbial population as functions of concentrations of glucose and galactose, respectively. It is apparent from these specific growth rate curves, shown in the inset of Figure 66, that an initial glucose concentration of 200 mg/ℓ can effect a growth rate of 0.68/hr compared to 0.60/hr for an initial galactose concentration of 200 mg/ℓ. Clearly, the organisms would find it profitable to grow on glucose alone, since this substrate allows a higher growth rate of 0.68/hr which is almost equal to the maximum of 0.683/hr. Glucose should be used exclusively until a growth rate of 0.60/hr is reached, at which point galactose



BATCH SYSTEM I: LOW $k^m$, LOW K AND $X_0^{ga}/X_0^g = 1$

| CURVE | UPTAKE OF | IN PRESENCE OF | EQUATION | $X_0^g$ (mg/l) | $X_0^{ga}$ (mg/l) | $X_0^o$ (mg/l) |
|---|---|---|---|---|---|---|
| 1 | Glucose | Galactose | 169 | 200 | 200 | 10 |
| 2 | Glucose | Galactose | 170 | 6.2 | 200 | 120 |
| 3 | Galactose | Glucose | $X_\theta^{ga} = X_0^{ga}$ | 200 | 200 | 10 |
| 4 | Galactose | Glucose | 171 | 200 | 6.2 | 120 |
| 5 | Galactose | Glucose | 169 | ≈0 | ≈194 | ≈124 |

LEGEND

- ——— GLUCOSE ($k^m = 0.683$/hr, $K = 0.8$ mg/l)
- - - - - - - GALACTOSE IN PRESENCE OF GLUCOSE ($k^m = 0.604$/hr, $K = 0.9$ mg/l)
- — — — GALACTOSE ALONE ($K = 3.9$ mg/l, $k^m = 0.387$/hr)

SUBSTRATE CONCENTRATION, $X_\theta^{ga}$ OR $X_\theta^g$ (mg/l) ⟶
200
160
120
80
40
0
k=0.68
0
1
2
3
4
5
TIME, $\theta$ (hours) ⟶
①
②
③
④
⑤
$\Delta\theta \approx 3$ min
$X_\theta^g = 6.2$
k=0.6
SPECIFIC GROWTH RATE, k, hr$^{-1}$
0.8
0.4
0
k=0.68
k=0.60
$X^g$
$X_0^g = X_0^{ga} = 200$
SUBSTRATE CONCENTRATION (mg/l)

FIGURE 66. THEORETICAL CURVES OF SEQUENTIAL UTILIZATION OF GLUCOSE AND GALACTOSE BY BATCH CULTURES OF SLOW GROWERS CHARACTERIZED BY LOW MAXIMUM SPECIFIC GROWTH RATE AND LOW SATURATION CONSTANT.

assimilation may be initiated. Exclusive utilization of glucose (Curve 1, Figure 66) for growth and energy of maintenance is described by Equation 169, the derivation of which is to be found in Appendix XV.

$$X_\theta^s = X_o^s - \frac{X_o^o(k^m+mY)}{Yk^m}\left[e^{k^m\theta}-1\right] \qquad (169)$$

and

$$k = \frac{k^m X_\theta^s}{K+X_\theta^s} \qquad (52)$$

where $X_\theta^s$ = substrate (glucose or galactose) concentration at any time, $\theta$

$X_o^s$ = initial substrate concentration in batch

$X_o^o$ = initial organism concentration in batch.

Galactose uptake is prevented during exclusive utilization of glucose presumably because the galactose premeases would be inhibited by a feedback mechanism discussed earlier, or repressed by glucose catabolites, depending on whether the permeases are constitutive or inducible in nature. In the absence of any galactose transport, there cannot be any intracellular galactose to stimulate the galactose operon for synthesis of the three galactose enzymes. The glucose concentration and the zero time for galactose assimilation (corresponding to k = 0.60/hr) were computed from Equations 52 and 169 to be 6.2 mg/ℓ and 3.7 hours, respectively. From $\theta$ = 3.7 hours, glucose and galactose should be concurrently assimilated. The concentrations of these two sugars at different points in time during concurrent utilization given by Equations 170 and 171, provided, of course, that glucose accounts for the cell yield and that galactose accounts for the energy of maintenance.

$$\ln X_{\theta}^{g} = \ln\left[\left(\frac{X_{o}^{o}U_{g}'+X_{o}^{g}-X_{\theta}^{g}}{U_{g}'}\right)\frac{X_{o}^{g}}{X_{o}^{o}}\right]+\left[\frac{X_{o}^{o}U_{g}'+X_{o}^{g}}{K_{g}}\right]\ln\frac{X_{o}^{o}U_{g}'+X_{o}^{g}-X_{\theta}^{g}}{X_{o}^{o}U_{g}'} - \frac{(X_{o}^{o}U_{g}'+X_{o}^{g})k_{g}^{m}\theta}{K} \qquad (170)$$

$$X_{\theta}^{ga} = \frac{k_{g}^{m}K_{ga}'X_{\theta}^{g}}{k_{ga}^{m'}K_{g}+X_{\theta}^{g}(k_{ga}^{m}-k_{g}^{m})} \qquad (171)$$

or

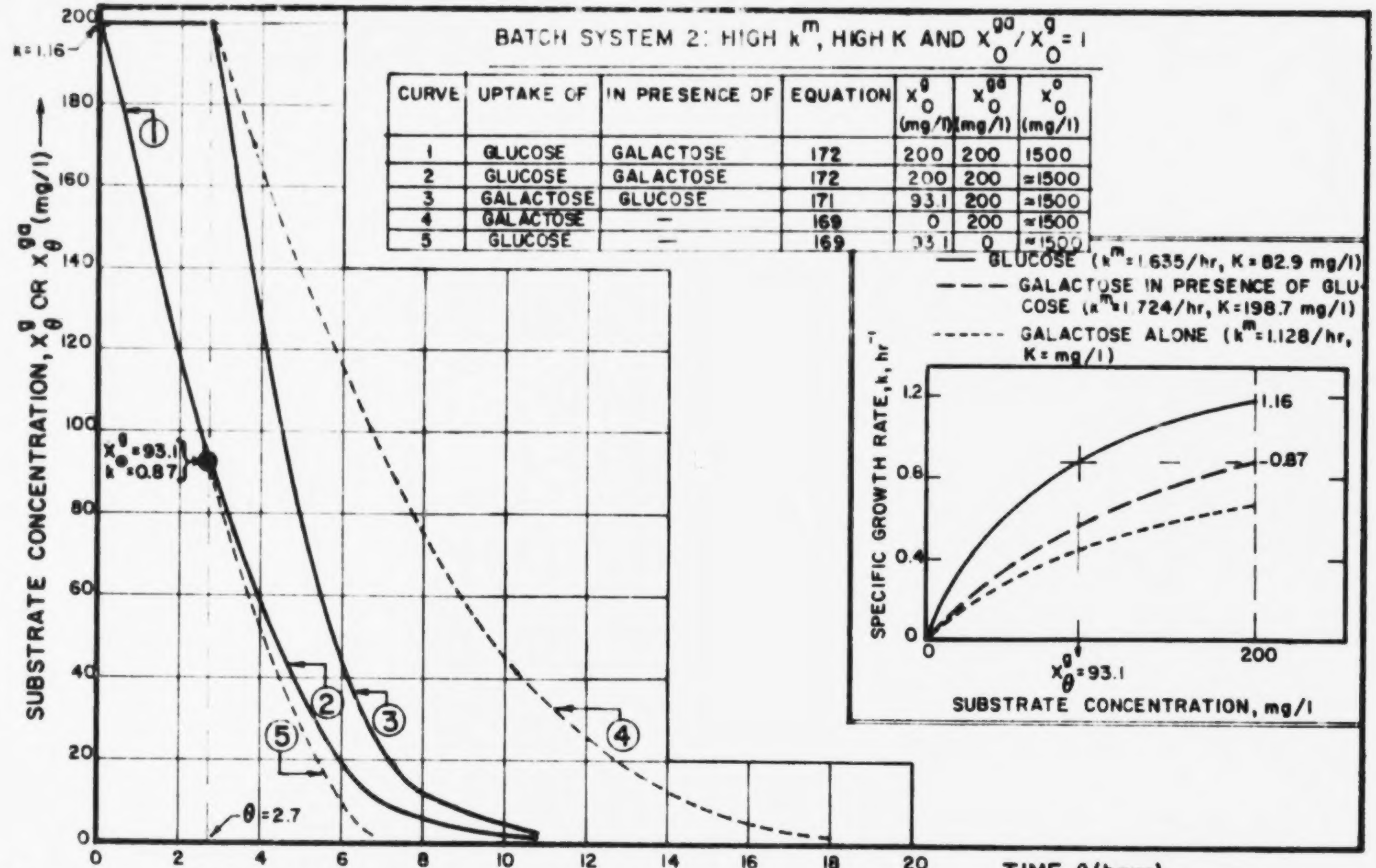
$$X_{\theta}^{ga} = \frac{k_{g}^{m}GX_{\theta}^{g}}{g^{m}K_{g}+X_{\theta}^{g}(g^{m}-k_{g}^{m})} \qquad (171A)$$

Derivation of Equation 170 is presented in Appendix XV. Equation 171 is similar in form to Equations 168 and 168A. It is apparent from Curve 1 of Figure 66 that glucose will be exhausted a very short time after the onset of galactose assimilation. Therefore, the second phase is characterized by exclusive utilization of galactose as shown by Curve 5 in Figure 66. Exclusive utilization of galactose was computed by using Equation 169. It may be pointed out that exclusive use of the two competing substrates in two separate phases would result in diauxie. The relative configurations of the specific growth rate curves of this batch system, as defined by the constants $k_{g}^{m}$, $K_{g}$, and $k_{ga}^{m'}$, $K_{ga}'$, are such that diphasic substrate utilization is almost inevitable excepting at very low concentrations of the substrates. The batch system used by Stumm-Zollinger (216-218) and Gaudy and his co-workers (211-214) was very similar to the theoretical system considered herein; as would be expected in light of the above analysis, the courses of sequential utilization of substrates observed by these investigators were very similar to those of Figure 66. Thus, sequential utilization of interacting substrates and diauxie are peculiar to a batch culture system employing substrates which are dissimilar in terms of the maximum specific growth rates, but similar in that they have very low values of the saturation constants.

The second batch system considered is one in which fast growing dominants (similar to population Type B), characterized by higher values of $\underline{k^m}$ and $\underline{K}$, assimilate glucose and galactose fed at initial concentrations of 200 mg/ℓ. In this system, glucose is utilized exclusively until its concentration is depleted to 93 mg/ℓ at which point both sugars can effect the same specific growth rate, and as such galactose assimilation can be initiated. It should be noted that the time lag for initiation of galactose utilization is much shorter than that of the first batch system with slow growers. The uptake patterns of glucose and galactose and the appropriate equations that describe the courses of utilization are presented in Figure 67. The solid curves represent the theoretical course of utilization of the interacting substrates. Exclusive utilization of glucose described by Curve 1 (Figure 67) is given by Equation 172 applicable to cultures of high saturation constants (see Appendix XV for derivation).

$$\theta = \frac{Y}{X_o^c(k^m+mY)}\left[(X_o^c-X_\theta^s) + \frac{K\{m-(k^m+mY)\}}{(k^m+mY)}\ln\left\{\frac{mYK+(k^m+mY)X_\theta^s}{mYK+(k^m+mY)X_\theta^s}\right\}\right] \qquad (172)$$

The dashed curves, 4 and 5, of Figure 67, respectively, represent the

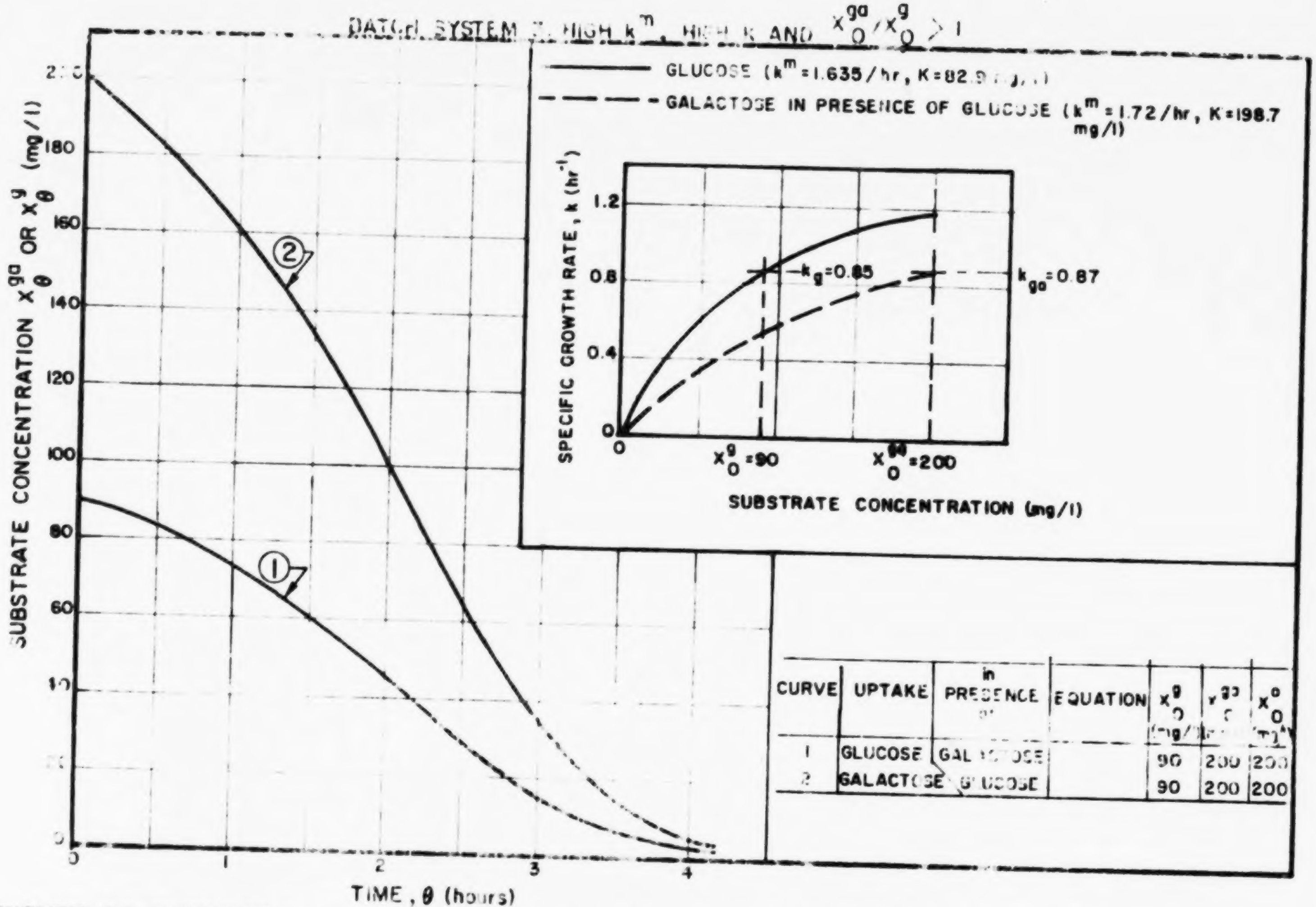


| CURVE | UPTAKE OF | IN PRESENCE OF | EQUATION | $X_0^g$ (mg/l) | $X_0^{ga}$ (mg/l) | $X_0^o$ (mg/l) |
|---|---|---|---|---|---|---|
| 1 | GLUCOSE | GALACTOSE | 172 | 200 | 200 | 1500 |
| 2 | GLUCOSE | GALACTOSE | 172 | 200 | 200 | ≈1500 |
| 3 | GALACTOSE | GLUCOSE | 171 | 93.1 | 200 | ≈1500 |
| 4 | GALACTOSE | — | 169 | 0 | 200 | ≈1500 |
| 5 | GLUCOSE | — | 169 | 93.1 | 0 | ≈1500 |

FIGURE 67. THEORETICAL CURVES OF SEQUENTIAL UTILIZATION OF GLUCOSE AND GALACTOSE BY A BATCH CULTURE OF FAST GROWERS CHARACTERIZED BY HIGH MAXIMUM SPECIFIC GROWTH RATE AND HIGH SATURATION CONSTANT.

uptake patterns of galactose and glucose as sole substrates and in the absence of interaction; these curves are presented to depict the impact of substrate interaction on batch kinetics. Comparisons of Curve 2 with 5 and Curve 3 with 4 reveal that the presence of galactose may somewhat retard glucose uptake, but glucose substantially enhances the rate of galactose utilization. It may be recalled that similar conclusions concerning the stimulatory effect of glucose were reached earlier during discussion of data on uptake of glucose-galactose mixtures by continuous cultures.

Figure 68 illustrates the theoretical uptake curves of glucose and galactose in still another batch system, also employing fast growers like those used in the system of Figure 67. It differs only in that the ratio of initial concentrations of galactose and glucose, $X_o^{ga}/X_o^g$, is considerably higher than unity. At the initial concentrations selected for analysis, namely, $X_o^{ga} = 200$ mg/ℓ and $X_o^g = 90$ mg/ℓ, both glucose and galactose can support about the same specific growth rate and would, therefore, be assimilated concurrently from the time of inoculation of the batch. Concurrent utilization of glucose and galactose in batch has been reported by Hernandez (219) using a $X_o^{ga}/X_o^g$ ratio of 1.73 compared to 2.22 used for the theoretical curves of Figure 68. Concurrent assimilation of glucose and sorbitol in batch cultures were also observed by Gaudy, et al. (213). Usually glucose and sorbitol give rise to diauxie, but serial subculturing for about 21 days provided a culture (referred to as "old" cells by Gaudy, et al.) which did effect simultaneous uptake of these substrates. It is interesting to note that the seed, which effected simultaneous uptake of glucose and galactose in the investiga-



| CURVE | UPTAKE | in PRESENCE of | EQUATION | $x_0^g$ (mg/l) | $x_0^{ga}$ (mg/l) | $x_0^o$ (mg/l) |
|---|---|---|---|---|---|---|
| 1 | GLUCOSE | GALACTOSE | | 90 | 200 | 200 |
| 2 | GALACTOSE | GLUCOSE | | 90 | 200 | 200 |

FIGURE 68. THEORETICAL CURVES OF CONCURRENT UTILIZATION OF GLUCOSE AND GALACTOSE BY A BATCH CULTURE OF FAST GROWERS CHARACTERIZED BY HIGH MAXIMUM SPECIFIC GROWTH RATE AND HIGH SATURATION CONSTANT.

tions of Hernandez (219), was also obtained after a series of subculturing of a heterogeneous seed. Inasmuch as the biokinetic properties of the dominants used by Hernandez (219) and Gaudy, et al. (213) in batch studies were not given by these authors, it is possible that serial subculturing resulted in a culture of fast growers which could cause concurrent uptake of the interacting substrates, as shown for Batch System 3 of Figure 68.

The preceding discussion emphasizes that substrate interaction and the extent thereof are functions of the biochemical properties of the organism-substrate system as well as the relative concentrations of the interacting substrates. Exclusive utilization of interacting substrates in separate phases, intervened by a negligibly short period of concurrent utilization, is the characteristic of a batch (or plug flow) culture with very low $K$ and low $k^m$, such as that of Figure 66. For a batch culture characterized by high $K$ and high $k^m$, the interaction is not severe because the period of exclusive utilization of the preferred substrate is shortened considerably compared to the system with low $K$ and $k^m$. Finally, with a high ratio of initial concentrations of the less preferred and the preferred substrate and a culture having high $K$ and $k^m$, it is possible to completely avoid sequential utilization.

It is to be noted that the theoretical batch assimilation curves of Figures 66, 67, and 68 were developed for the batch growth of a seed population of slow or fast growers. If the starter population in batch reactors contains a mixture of slow and fast growers, then the courses of assimilation of the competing substrates would depend on the relative concentrations of the slow and fast growers at the time of initiation of the batch culture. The differential equations for the substrate utilization curves then become complicated necessitating predictions of performance by computer analysis.

*Interacting Substrates in Continuous Flow Reactors*. In continuous flow systems of this research, the sequential type of utilization was not possible since the slow growers with low $\underline{K}$ and $\underline{k}^m$, which make such a pattern of uptake possible, cannot be maintained at higher dilution rates. With increased dilution rates, the reactor population becomes increasingly dominated by fast growers having high $\underline{K}$ and $\underline{k}^m$; the continuous culture system thus approaches the Batch System 3 of Figure 68. Consequently, simultaneous uptake of glucose and galactose should be effected as was actually observed.

In the continuous culture experiments of this research, the ratio of the concentrations of influent galactose and glucose was maintained between 1.05 to 1.23. Since lower ratios of $X_o^{ga}/X_o^g$ tend to favor sequential assimilation of glucose and galactose notwithstanding the presence of fast growers with high $\underline{K}$ and $\underline{k}^m$, it is quite probable that galactose assimilation may be avoided in continuous flow reactors if the aforementioned ratios of influent concentrations fall below 1.00. This postulate remains to be experimentally verified. However, Equation 168 may be used to specify conditions under which glucose would prevent galactose utilization in continuous flow reactors. For instance, if the galactose concentration, $X_o^{ga}$, is lower than the $X_o^{ga'}$ predicted from Equation 168 and a given reactor glucose concentration of $X_1^g$, no galactose uptake can be effected. Thus, in Run 7 with a glucose-galactose mixture, $X_o^{ga}$ nearly equaled $X_1^{ga'}$ (see Table 9): galactose transport was almost completely inhibited by glucose.

The discussion of this section was included to reconcile the apparent contradictions between the results of several investigators of substrate interactions. The observation of sequential as well as the concurrent uptake of interacting substrates in batch processes and simultaneous utilization of these substrates in continuous flow processes are consistent with the following hypothesis that the pattern of substrate removal (sequential versus concurrent) and the kinetics thereof are determined by: (a) the biokinetic properties of the organism-substrate system employed, (b) the ratio of concentration of the interacting substrates in the feed solution, and (c) the fluid dynamics of the culture system. The first factor is related to physiology and biochemistry of the system, while the second and the third factors are chemical and physical in nature, respectively.

## Sanitary Engineering Significance

Continuous Processes. The results of the research were suggestive of the fact that, in continuous aerobic processes, the growth kinetic properties of the microbial populations undergo continual change in response to the changes in detention time. Sufficient theoretical and experimental evidence indicated that variation of the biokinetic capabilities was a manifestation of the changes in the physiological capabilities and species composition of the reactor populations. At high flow-through rates, fewer species characterized by high maximum specific growth rates and saturation constants prevailed. In high rate aerobic processes, one may therefore anticipate the predominance of fewer species especially suited to grow in a very hydraulically selective environment. If substrates in which the fast growing dominants grow poorly or not at all are introduced, then the process might perform poorly. Whether organism recycle will alleviate the problem remains a moot question and is a subject of further investigation. Conventional processes operated at higher detention times maintain a far more heterogeneous population and any sudden increase in the concentration of any incoming organic substrate can be attacked by a species which heretofore were present in relatively smaller cell concentrations. Therefore, a high rate process is suitable where the composition of the incoming waste remains uniform in time, whereas a conventional (low-rate) process is suitable for waste whose chemical composition changes with time. It appears that a low rate process can absorb shock loading better than the high rate process.

As detention time is increased, the physical and environmental conditions in a continuous reactor approach those of a batch reactor. The batch growth constants therefore may approximate the constants determined with continuous reactors operated at very high detention times. However, the inherent environmental characteristics of a batch reactor preclude the possibility of selection of the fast growing species and the use of batch constants for design of a high rate process would result in high detention times. The fast growing species, which make the high rate process functional, cannot be sustained in high enough mass concentrations at any detention time so designed. This would result in an operational efficiency lower than the design efficiency. Consequently, batch data should be used with caution and judgment for design of high rate processes.

This research has also shown that substrates known to yield diauxie in batch can be biodegraded concurrently at all detention times provided a high enough ratio is maintained between the concentrations of the less preferred and the preferred substrates. The results serve to demonstrate that the less preferred substrate does not necessarily have to go unassimilated in a reactor, as had been suggested by some investigators (216-218,211-214,220). However, it was also shown that, if the influent concentration of the less preferred substrate falls below the value determined by an equation such as Equation 168A, then it would pass through the reactor undegraded; the required degree of removal of this substrate can be effected, however, by lowering the hydraulic loading or treating this substrate component in a separate tank. The results also revealed that, in mixed substrates, one substrate may satisfy the carbon requirement while another may fulfill the energy needs. Sequential assimilation of interacting substrates is preferred in heterogeneous batch cultures as the hydraulics of the system are not conducive to population shifts. Long holding times would be needed to ensure removal of both substrates. The same conclusions apply for plug flow reactors in which a long detention time would be necessary to prevent leakage of the less preferred substrate. It appears that, for the same detention time, a completely mixed continuous-flow reactor would effect better removal of interacting substrates than would be possible in a plug flow reactor. This postulate is not based on detailed experimental or theoretical grounds.

From the viewpoint of process design, it is of some interest to have information on the fraction of substrate removal responsible for sludge production as well as the fraction which is biologically oxidized to $CO_2$ and $H_2O$ and other end products. It is also important to compare waste substrates in terms of their sludge yields, amenability for oxidative conversion to $CO_2$ and $H_2O$, and stabilization through microbial utilization. These may be best accomplished through the aid of graphs such as those of Figures 69, 70, 71, 72, and 73. The curves in the figures are defined by equations either derived earlier or as derived below.

In case of mixed substrates, where individual organics can be measured separately, sludge yields are to be expressed in terms of biomass per mass of COD removed. Appropriate equations for sludge yield in the above terms can be derived in the following manner.

From Equations 156 and 157:

$$\left[\frac{(X_o^{ga}-X_1^{ga})+(X_o^{g}-X_1^{g})}{X_1^{o}}\right] = (U'_{ga}+U'_{g}) + \theta_r[k'(U'_{ga}+U'_{g})+(m'_{g}+m'_{ga})] \qquad (173)$$

If glucose and galactose removal is measured in terms of COD, then

$$\frac{X_1^{o}}{(X_o^{g}-X_1^{g}) + (X_o^{ga}-X_1^{ga})} = Y^{oa}_{g-ga} \qquad (174)$$

where $Y^{oa}_{g-ga}$ = apparent yield of active solids in milligrams of active solids per mg of glucose and galactose COD removed.

Equation 173 plots as a straight line similar to those of Figures 44 and 47. The intercept of the line of best fit at $\theta_r = 0$ will be the reciprocal of the growth yield, $Y^{oa}_{gga}$. The coefficient of energy of maintenance in terms of the amount of COD used per unit quantity of biomass per unit of time can then be computed from the slope of the line. From Equations

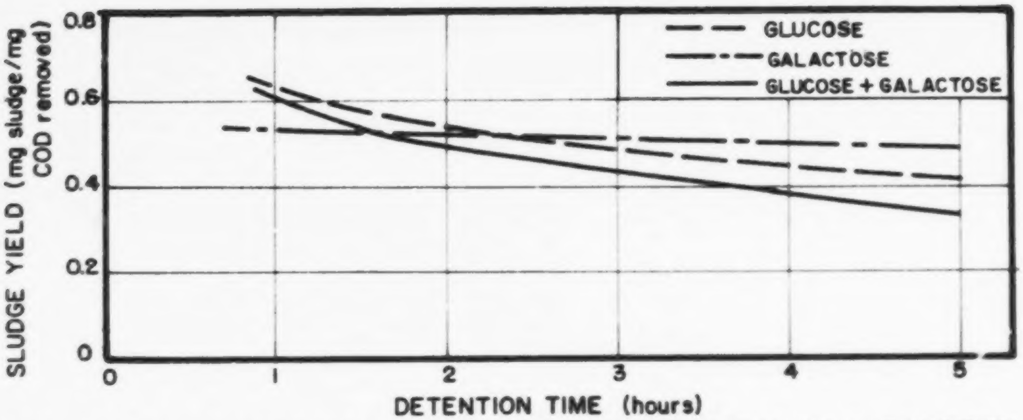


FIGURE 69. THEORETICAL CURVES OF SLUDGE YIELD AS A FUNCTION OF DETENTION TIME FOR CULTURES GROWN ON GLUCOSE, GALACTOSE AND GLUCOSE-GALACTOSE MIXTURES.

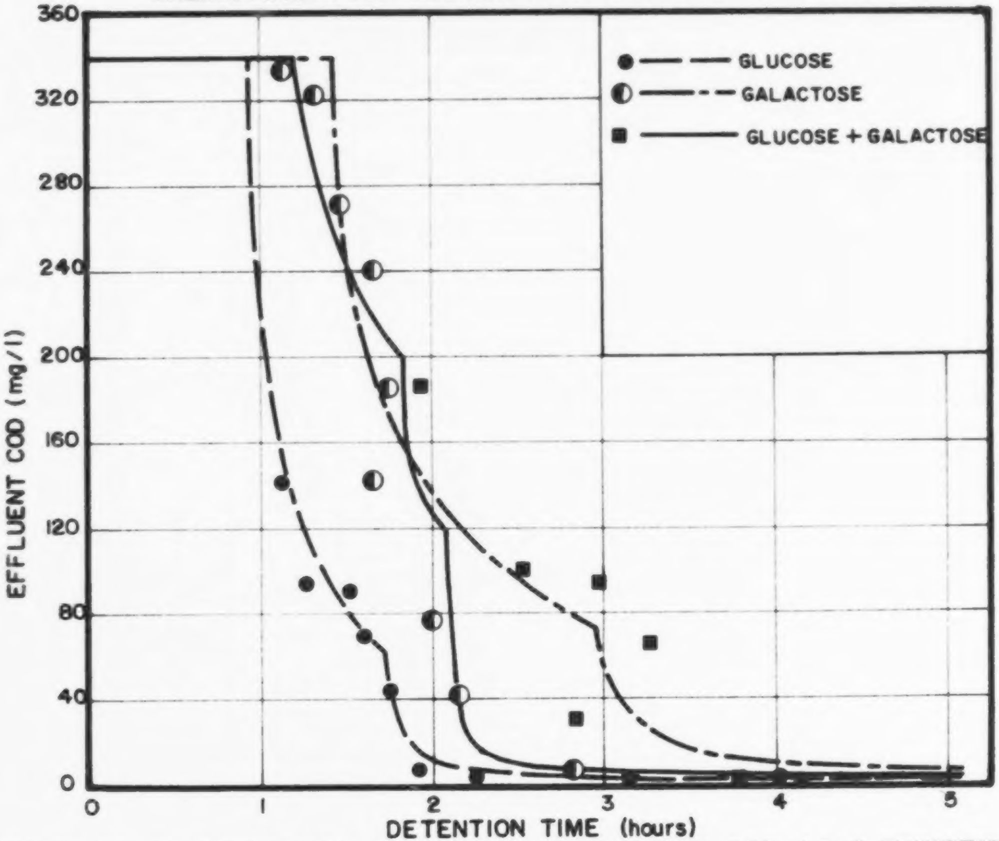


FIGURE 70. THEORETICAL CURVES OF EFFLUENT COD AS A FUNCTION OF DETENTION TIME FOR CULTURES GROWN ON GLUCOSE, GALACTOSE, AND MIXTURES OF GLUCOSE AND GALACTOSE.

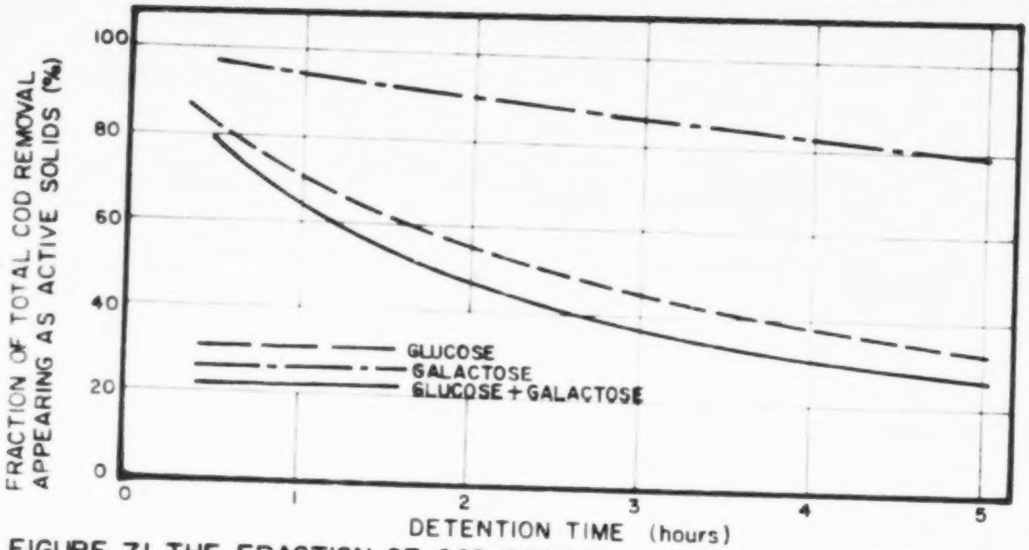


FIGURE 71. THE FRACTION OF COD REMOVAL USED FOR PRODUCTION OF ACTIVE SOLIDS FROM THE INDICATED SUBSTRATE AT VARIOUS DETENTION TIMES.

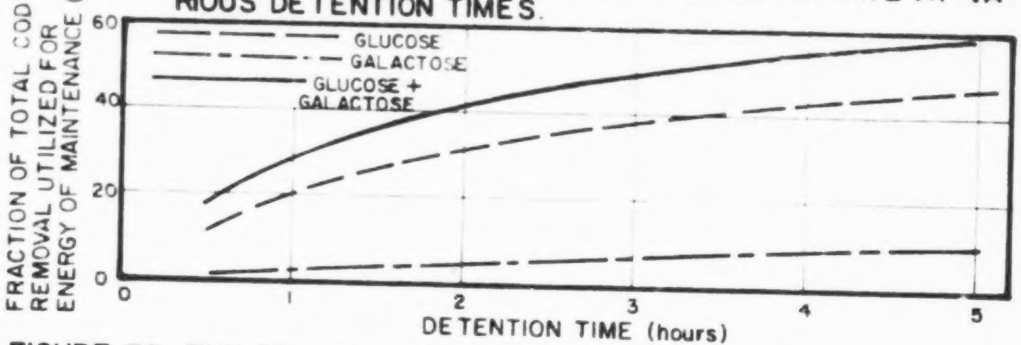


FIGURE 72. THE FRACTION OF COD REMOVAL USED FOR THE ENERGY OF MAINTENANCE OF BIOMASS GROWN FROM THE INDICATED SUBSTRATES AT VARIOUS DETENTION TIMES.

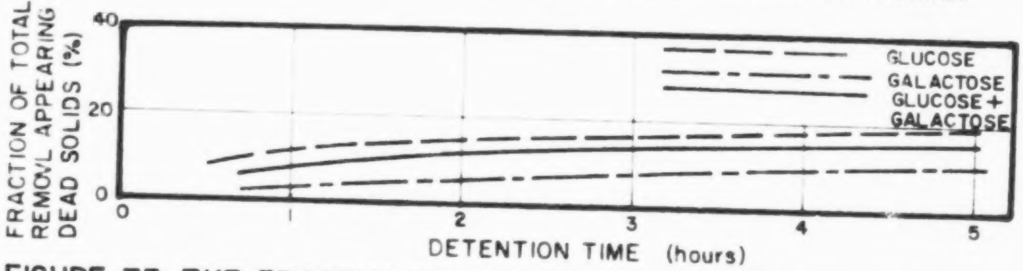


FIGURE 73. THE FRACTION OF COD REMOVAL USED FOR THE PRODUCTION OF BIOMASS WHICH DIE AT VARIOUS DETENTION TIMES OF GLUCOSE, GALACTOSE AND MIXED GLUCOSE-GALACTOSE FED CONTINUOUS FLOW REACTORS.

173 and 174 and Equation 93 of Chapter IV, and considering that $m'_g$ and $U'_{ga}$ are both equal to zero, one can easily derive the following equation for sludge yield in terms of total solids from mixed substrates of glucose and galactose:

$$Y^{o\,t} = \frac{(1+k'\theta_r)}{U'_g + m'_{ga}\theta_r + k'U'_g\theta_r} \qquad (175)$$

Figure 69 compares the sludge yields on the two individual sugars and their mixtures. Due to the greater energy requirements of the glucose and the glucose-galactose fed cultures, sludge yields were lower than that of the galactose fed cultures at detention times above 2.5 hours.

The curves of Figure 70, which were derived from Equation 64A and Curves 2 and 3 of Figures 45 and 49 and from Curves 1A and 1B of Figure 60, compare the effluent qualities at various detention times for the individual and mixed sugar substrates. It is probable that the multiple steps of the effluent COD curve of mixed substrate may be typical of reactors charged with complex substrates. Comparison of the effluent qualities at various detention times shows that the presence of the less preferred organic, such as galactose, causes deterioration of the quality of effluent from a reactor treating only glucose. Conversely, introduction of an efficient and preferred substrate such as glucose considerably improves the effluent quality of a reactor treating relatively inefficient substrates like galactose. The foregoing conclusions form the theoretical basis for ununiform effluent quality from reactors charged with wastes whose chemical composition varies with time.

It can be further concluded from comparison of the theoretical curves of Figure 70 that introduction of less preferred substrates does not appreciably affect effluent quality if the reactor is operated at higher detention times (above 2.5 hours for glucose and galactose). Rather, the inefficient substrate will have the effect of reducing sludge production (see Figure 69) thus minimizing sludge handling and disposal problems. Therefore, the high rate process is sensitive to the diauxic phenomenon while the conventional process is not.

Figures 71 through 73 show the fractional utilization of assimilated substrate, in terms of COD removal, for growth and derivation of energy through biological oxidation at different detention times in terms of COD removal. The fraction utilized for growth at any detention time can be further subdivided into: (a) the fraction which gives rise to the active cells, and (b) the fraction which would account for the growth of cells which eventually die. The fractions of assimilated single substrate that account for active biomass, dead cells, and energy of maintenance could be computed with the aid of Equations 95, 96, and 97 of Chapter IV. For mixed substrate, the following expressions, derived in a manner analogous to that for derivation of Equations 95, 96, and 97, were used.

Percentage of COD removal used for growth of active cells = $U'_g Y^{oa}_{gga}$ (176)

Percentage of COD removal used for supplying energy of maintenance by biological oxidation = $m'_{ga} \theta_r Y^{oa}_{gga}$ (177)

and

Percentage of COD removal used for growth of cell which remains as dead or inactive = $k' U'_g \theta_r Y^{oa}_{gga}$ (178)

Figures 71, 72, and 73 emphasize that, relative to a single substrate, a larger fraction of assimilated mixed substrate COD was biologically converted to $CO_2$ and $H_2O$, and a smaller fraction was converted to biomass. The significant conclusion to be made from the above discussions is that mixed substrate, even when it contains diauxie yielding organic components, is preferable to single substrate from the viewpoint of lower sludge production and higher percentage destruction of organics.

# CHAPTER IX

# CONCLUSIONS

From the experimental results and discussions of the previous chapters, the following conclusions were drawn.

## Continuous Process Kinetics for Utilization of Single Sugar Substrate

1. The specific growth rate, k, of the dominant and heterogeneous microbial population at any reactor detention time, $\theta_r$, was satisfactorily described by the following equation proposed by Monod:

$$k = \frac{k^m X_1^s}{K + X_1^s}$$

where $k^m$ = maximum specific growth rate ($hr^{-1}$)

$X_1^s$ = steady state substrate concentration (mg/$\ell$)

K = saturation constant for the organism-substrate system (mg/$\ell$)

The specific growth rate was shown to be related to the reactor detention time and the specific death rate, $k'$, by:

$$k = \frac{1}{\theta_r} + k'$$

2. A satisfactory measure of dead, inactive, and inert biological solids was obtained from the difference between the total solids concentration, $X_1^{o\,t}$, and the active biomass concentration, $X_1^o$. Therefore,

the following equation was derived to describe the specific death rate, $k'$:

$$k' = \frac{X_1^{o\,t} - X_1^o}{X_1^o \theta_r}$$

The specific death rate was constant for a given substrate, a direct function of the growth yield (or culture density) and substrate consumption and an inverse function of the extracellular substrate concentration.

3. The following equations were derived and can be used to describe the steady state organism and substrate concentration:

$$X_1^o = \frac{(X_o^s - X_1^s)Y}{1 + \theta_r k' + mY\theta_r}$$

$$X_1^s = \frac{K(1+k'\theta_r)}{\theta_r k^m - (1+k'\theta_r)}$$

where $X_o^s$ = substrate concentration in influent to reactor (mg/$\ell$)

$Y$ = true growth yield coefficient

$m$ = maintenance coefficient (hr$^{-1}$)

4. Measured dehydrogenase activities of the cultures gave a satisfactory estimate of the active biomass concentration, $X_1^o$. Dehydrogenase activities were linearly related to active biomass concentration, and the same relationship was valid for all specific growth rates during exponential growth on glucose or galactose. Glucose and galactose grown cultures exhibited dehydrogenase activities which were higher

than those exhibited by cultures grown on glucose-galactose mixtures.

5. The observed yield coefficient, $Y^{oa}$, measured in terms of active solids, decreased with increases in detention time. Growth yields from glucose and galactose were higher in continuous cultures than those observed in batch cultures.

6. Continuous cultures at different detention times exhibited different growth kinetic properties. The dominant microbial populations at detention times above 1.7 hours for the glucose substrate and 2.9 hours for the galactose substrate were characterized by $\underline{k}^m$ and $\underline{K}$ values which were lower than those of the population dominant below these detention times. (Accordingly, cultures dominant above these detention times were called fast growers. Detention times exceeding 1.7 hours for the glucose fed reactors and 2.9 hours for the galactose fed reactors were considered to be high detention times.)

7. The experimental data indicated that a slow growing microorganism, j, was eliminated in the presence of a fast growing microorganism, $\ell$, if:

$$\frac{k_j^m K_\ell - k_\ell^m K_j}{K_\ell - K_j} - k' < D$$

$$\frac{k_j^m K_\ell - k_\ell^m K_j}{k_\ell^m - k_j^m} < X_1^s$$

8. The heterogeneity of the continuous cultures decreased with decreases in detention time. In addition, the variabilities of the steady state substrate and organism concentration were related to the

degree of heterogeneity of the continuous culture and it decreased sharply with decreases of detention time.

9. The heterogeneous cultures at high detention times contained larger populations of Gram negative rods, members of the coliform group and free swimming protozoa.

10. Slime or attached growths in the reactor or on other solid surfaces proved to be a definite impediment to the attainment of steady states.

11. Glucose was a more efficient growth promoter than galactose because it could cause high cell yields, higher maximum specific growth rates (i.e., lower minimum generation times), and also because the organisms utilizing glucose had a lower saturation constant (i.e., higher affinity for this substrate).

## Kinetics of Utilization of Interacting Substrates

1. Glucose and galactose were simultaneously assimilated at all detention times above 1.15 hours and when the ratio of the influent concentrations of galactose and glucose remained between 1.05 to 1.23.

2. Glucose consumption accounted for the growth yields whereas galactose consumption accounted for the energy of maintenance of the cultures. The yield and maintenance coefficients of the cultures fed with mixed substrate were higher than those of the cultures grown on the individual substrates.

3. The following equations were derived and found capable of describing glucose and galactose uptake by cultures fed with mixed sugars:

$$\frac{X_o^g - X_1^g}{X_1^o} = U_g' + k'U_g'\theta_r$$

$$\frac{X_o^{ga} - X_1^{ga}}{X_1^o} = m_{ga}'\theta_r$$

where $U_g'$ = reciprocal of growth yield coefficient (mg glucose/mg of active biomass)

$m_{ga}'$ = maintenance coefficient in terms of galactose uptake (mg galactose/mg of active biomass-hours)

4. The specific growth rates of cultures grown on glucose-galactose mixtures were controlled by the glucose concentration during simultaneous utilization of the two sugars.

5. Cultures grown on glucose-galactose mixtures had the same $k^m$ and $K$ as observed for the glucose grown cultures.

6. Glucose uptake rate (mg/ℓ-hr) by the culture grown on glucose-galactose mixture at any detention time was not significantly different from that observed for the glucose grown culture at the same detention time.

7. Galactose uptake rate (mg/ℓ-hr) increased while the *specific galactose uptake rate* (mg of galactose/mg of biomass-hr) decreased in the presence of glucose. Increased galactose uptake rate was attributable to higher yield and maintenance coefficients for the cultures grown on mixed sugars.

8. Increased galactose removal rate by glucose-galactose fed cultures caused the steady state galactose concentrations to be lower than those observed with the galactose grown cultures. The following

equation was derived to express steady state galactose concentration, $X^{ga'}$, as a function of the steady state glucose concentration, $X_1^g$:

$$X_1^{ga'} = \frac{k^m G X_1^g}{g^m K + X_1^g(g^m - k^m)}$$

where $\underline{G}$ and $\underline{g^m}$ are *galactose uptake parameters* related to the Michaelis constant and the maximum velocity, respectively, of the rate controlling enzymatic step in galactose metabolism.

Galactose remained unassimilated if the influent galactose concentration was less than $X_1^{ga'}$ given by the equation. In addition, for simultaneous assimilation of glucose and galactose, the ratio of influent concentrations of galactose and glucose must be more than unity.

9. In the presence of glucose, the *specific galactose uptake rate* decreased as the steady state glucose concentration increased. In light of the galactose permeation model and the cellular regulatory mechanisms proposed by various researchers, the *specific galactose uptake rate* in the presence of glucose was considered regulated by (noncompetitive) feedback inhibition and/or catabolite repression of the galactose permease.

10. The galactose uptake parameter, G, was lower than the saturation constant of slow growers when galactose was the sole substrate thereby indicating an increased affinity of the slow growers for galactose at very low concentrations in the presence of glucose.

## The Role of Biochemical and Environmental Factors in Determining the Mode of Uptake of Competing Substrates

1. Based on the observed growth kinetic characteristics of slow and fast growers in continuous cultures, the following could be theorized regarding the mode and kinetics of uptake of competing substrates by batch cultures.

(a) Glucose and galactose can be sequentially removed by batch cultures of slow growers; glucose will be preferentially assimilated in the first phase. Exclusive utilization of either substrate is given by the following equation:

$$X^s_\theta = X^s_o - \frac{X^o_o(k^m+mY)}{Yk^m}\left[e^{k^m\theta}-1\right]$$

where $X^s_\theta$ = concentration of substrate at any time, $\theta$

$X^o_o$ = initial organism concentration in batch culture

$X^s_o$ = initial substrate concentration in batch culture

(b) Glucose and galactose can be concurrently assimilated by batch cultures of fast growers if the ratio of initial concentrations of galactose and glucose exceeds a limiting value (e.g., 2.20 for the fast growers during this research). The following equations were derived to describe the glucose and galactose concentrations as functions of time:

$$\ln X^g_\theta = \ln\left[\left\{\frac{X^o_oU'_g+X^g_o-X^g_\theta}{U'_g}\right\}\right] + \frac{X^o_oU'_g+X^g_o}{K_g}\ln\left[\frac{X^o_oU'_g+X^g_o-X^g_\theta}{X^o_oU'_g}\right] - \frac{(X^o_oU'_g+X^g_o)k^m_g\theta}{K}$$

$$X^{ga}_\theta = \frac{k^m_gGX^g_\theta}{K_gg^m+(g^m-k^m_g)X^g_\theta}$$

2. Sequential uptake of glucose and galactose was not possible in continuous cultures because the slow growers favoring phasic uptake at higher specific growth rates were displaced by fast growers capable of simultaneous uptake of these substrates.

3. In mixed substrate systems, the substrate having the lowest $\underline{K}$ and capable of bringing about the highest $\underline{k}^{m}$ and $\underline{Y}$ becomes growth controlling and will be preferentially assimilated as long as its concentration can support the highest possible specific growth rate. The kinetics of uptake of the preferred substrate remain the same as in the absence of any competing substrate. However, the kinetics of uptake of the less preferred substrate are modified in the presence of the preferred substrate.

4. The apparent contradictions of conclusions reported by various investigators of the sequential uptake phenomenon are reconcilable in light of the following hypothesis:

> The mode and kinetics of assimilation (sequential or concurrent) of competing substrates are determined by the nature of the specific growth rate functions with reference to the individual substrate concentrations, the relative configurations of the resultant specific growth rate curves of the various substrates, the ratio of concentrations of the substrates, and the type of flow model used for the culture system.

## Conclusions of Engineering Significance

1. Active biomass concentration in heterogeneous biological processes can be measured by correlating dehydrogenase activity with the solids concentrations for a specific organism-substrate system.

2. A high rate aerobic process contains a dominant population of fast growers whereas a low rate process contains many species with a prevalance of slow growers.

3. Preferential utilization of glucose from glucose-galactose mixtures is not possible if a continuous reactor is operated at detention times higher than two hours.

4. Compared to a single substrate influent, a larger fraction of the assimilated mixed substrate is biologically oxidized and a smaller fraction is synthesized into biomass.

5. The quality of the effluent from an aerobic reactor treating an efficient substrate like glucose deteriorates if a less efficient substrate like galactose is introduced into the influent. However, if the reactor is operated at a sufficiently high detention time (e.g., four hours for glucose-galactose systems), no significant adverse effect will be observed.

# CHAPTER X

## RECOMMENDATIONS

A very significant observation made during this research was the phenomenon of population dynamics in completely mixed continuous flow reactors. The kinetics of growth and substrate removal as well as the nature of substrate interaction and the kinetics of uptake of interacting substrates were greatly influenced by the nature of population shift that resulted from the shifting of the reactor detention time. This particular aspect of continuous processes and its effect on process kinetics has received little attention from researchers in Sanitary Engineering. Further research should be undertaken to delineate those biochemical and environmental factors which may cause the occurrence of such population shifts. The investigation should include in its purview the isolation and identification of the dominant species at selected detention times and the study of the growth kinetic properties of individual dominant species. The information should prove invaluable in constructing realistic mathematical models for prediction of the behavior of continuous flow biological processes.

The rational models describing the kinetics of substrate removal are based on the kinetics of growth. These models are satisfactory for predicting the disappearance of substrates that are growth limiting. This investigation has revealed that, in the presence of several potentially growth limiting carbon and energy sources, growth is controlled by the substrate capable of effecting the maximum cell yield and reproduction rate. The other carbon and energy sources may serve as supplementary nutrients for energy or other requirements. It has been shown that the uptake of the secondary carbon and energy source is, like the uptake of the primary substrate, a hyperbolic function of the specific growth rates: the function included uptake parameters analogous to the growth kinetic parameters $\underline{k}^m$ and $\underline{K}$. However, the model was satisfactory for the case in which the secondary substrate accounted for the energy of maintenance and when the secondary substrate remained in concentration exceeding the concentration of the growth controlling substrate. Additional continuous culture studies may be undertaken to test the validity of the hyperbolic relationship between specific growth rate and concentration of the secondary substrate for different ratios of influent substrate concentration. The assertion that secondary substrates like galactose would pass through a chemostat type reactor unmetabolized at low ratios of influent concentrations of galactose and glucose requires further experimental verification.

It has been hypothesized that sequential or concurrent utilization of competing substrates in batch or plug flow reactors is primarily determined by the growth kinetic characteristics of the microbial population and the ratio of initial concentration of the interacting substrates. Therefore, microorganisms characterized by low uptake parameters ($k^m$ and $K$) for glucose and galactose are expected to effect sequential removal of these sugars if the initial glucose concentration is above about 10 ppm. On the other hand, concurrent assimilation of glucose and galactose may be brought about by a microbial population having higher uptake parameters if the feed concentrations of galactose exceed that of glucose by more than 25 percent. Confirmatory investigations should be conducted in batch reactors with various ratios of initial concentrations of the two sugars. The batches should be inoculated with microbial populations that dominate at various detention times in a glucose-galactose fed chemostat type reactor.

It has been shown that the dehydrogenase test is a simple technique with good theoretical basis for furnishing a measure of concentration of active biological solids. Dehydrogenase activity was proportional to solids concentration and the constant of proportionality was independent of growth rates for a given microbial population and substrate composition. However, detailed investigations should be carried out to study the effects of varying population and substrate compositions on the relationship between the dehydrogenase activity and the active solids concentration.

Despite the fact that the substrate conversion rate increased with decreases in detention time, there are some disadvantages of reactor operation at low detention times of a high rate process. These are:

1. unacceptable effluent quality in terms of BOD or COD;
2. formation of volatile acids and possibly other organic intermediary products;
3. a greater possibility of diauxic type utilization of competing substrates in mixed substrate systems; and
4. vulnerability of the high rate process to changes of chemical and/or physical environments of the reactor (i.e., vulnerability to shock loading).

In addition, the continuous culture equations commonly used have to be revised in case of formation of volatile acids or other products of mixed metabolism. Much more research should be directed towards a clearer understanding of the behavior, kinetics, and limitations of the aerobic high rate process from the viewpoint of the applicability of this process for waste stabilization.

# APPENDICES

# APPENDIX I

## DESIGN OF THE CULTURE VOLUME FOR THE CONTINUOUS FLOW REACTOR

The volume of the continuous flow reactor for research purposes should be chosen after consideration of the advantages and disadvantages of selecting unduly large or small reactor volumes.

The advantage of a large reactor volume is that a large number of samples can be withdrawn without significantly affecting the culture volume, the detention time, and the rate of oxygen transfer. The disadvantages of a large reactor are:

a) need for larger quantities of substrates and nutrients;
b) need for larger feed reservoirs;
c) pumping and handling of large quantities of feed solutions;
d) larger space requirement; and
e) higher cost of reactor, feed reservoirs, appurtenances, chemicals, labor, and power.

For reasons of economy, the smallest possible reactor volume should be selected. However, the volume should be large enough to accommodate the agitation device, baffles, a thermometer, a DO probe, a pH probe, and any other essential fittings or equipment. Operation of a small reactor at large detention times requires very low rates of steady flow of nutrients which are difficult to attain. Operation in such situations is accomplished by discontinuous addition of nutrients in discrete drops at regular intervals. Such a practice is undesirable as it often leads to fluctuations of substrate and organism concentrations in the reactor; the magnitude and amplitude of the fluctuations depend on the concentration differential between the drops of the influent and the reactor contents. At large detention times of a small reactor, the frequency of the concentration swing will be lower, while its amplitude will be higher, thus resulting in a highly oscillatory steady state. Since heterogeneous cultures tend to cause oscillatory steady states, selection of a small reactor would only aggravate this problem.

With a small reactor not more than five percent of the reactor volume should be withdrawn in the samples during the period of one detention time; such a practice ensures that the detention time or the oxygen transfer rate is not significantly affected due to the withdrawal of reactor samples. Samples should be collected from the effluent if the frequency of sampling is high and/or the sample volumes are large. If samples are collected from the effluent, then the reactor volume should be so designed that there is negligible fluctuation in the steady state concentration during the period required to collect the desired volume of a sample. Perret (319) has derived the following equations for the minimum reactor volumes required to limit the amplitude and frequency of fluctuations of reactor substrate concentrations to selected values of the same.

$$V_{R_1} = \frac{v_d X_o^S}{\alpha X_1^S} \qquad (179)$$

$$V_{R_2} = \frac{v_d \beta}{k \ln 2} \qquad (180)$$

$$V_{R_3} = \frac{v_s}{k\theta_s \ln 2} \qquad (181)$$

where $V_{R_1}$ = minimum volume of reactor required for limiting the amplitude of fluctuation of substrate concentration to $\alpha X_1^S$

$V_{R_2}$ = minimum reactor volume required for limiting the frequency of fluctuation of substrate concentration to the desired value

$V_{R_3}$ = minimum reactor volume required in order that fluctuations of substrate concentration are negligible during the selected period, $\theta_s$, of collection of sample of desired volume, $v_s$

$v_d$ = volume of a drop of feed solution

$X_o^S$ = concentration of substrate in the influent

$X_1^S$ = concentration of substrate in the reactor

$\alpha$ = a factor to be selected

$\beta$ = allowable frequency of fluctuation of reactor substrate concentration per unit time

$k$ = specific growth rate

$v_s$ = volume of sample collected from the effluent

$\theta_s$ = time of collection of sample from the effluent.

The following values of the minimum culture volume were computed based on the anticipated values of the variables at high detention time ($v$ = 0.05 ml, $X_o^S$ (max) = 400 mg/ℓ, $X_1^S$ (min) = 0.5 mg/ℓ, $k$ (min) = 0.2/hr,

$v_s = 20$ ml, and $\theta_s = 2$ min) and the allowable limits of fluctuation of substrate concentration of $\alpha = 0.025$ and $\beta = 60/\text{hr}$:

$V_{R_1} = 1.6$ liters

$V_{R_2} = 21.6$ milliliters

$V_{R_3} = 4.4$ liters

The selected culture volume should be at least equal to the maximum of the three volumes computed above. A minimum reactor volume of 4.5 liters was chosen.

# APPENDIX II

# HYDRAULIC ANALYSIS OF THE CONSTANT HEAD DEVICE EMPLOYED FOR THE DELIVERY OF FEED SOLUTIONS TO THE CONTINUOUS FLOW REACTOR

Under the conditions of operation of the Lute as outlined in Chapter V, pressure at Point 4 of Tube A (see Figure 15A) is

$$p_4 = wd_a = \text{constant},$$

since, $d_a$, is kept constant for a particular run. Upon sealing the feed reservoir from the atmosphere, either of the following two situations could be encountered depending on the relative depths of the Tubes A and C (see Figure 15A) below the water levels:

(a) when $d_a > d_c$, bubbles of air would be drawn inside the reservoir until $p_4 = p_3$ in which condition bubbles would escape in the Lute at point 4 and the meniscus in Tube C would remain at Point 3; or

(b) if $d_a < d_c$, the meniscus in Tube C would remain above Point 3, but could be brought down to this level by draining some solution by opening Stopcock C and thus lowering the air pressure above the solution in the reservoir. In any case, when the meniscus was at Point 3, the following relationship held:

$$p_3 = p_4 = wd_a = \text{constant} \qquad (182)$$

If Tube B were full of solution and unclamped, then flow could or could not commence depending on the elevation of Point 1. When the elevation of the free end of Tube B was such that there was flow of nutrients, from Bernoulli's theorem

$$p_1/w + z + h_f = p_2/w \tag{183}$$

where $h_f$ = friction head in Tube B of length $\ell$.

It is also true that

$$\frac{p_2}{w} = \frac{p_2}{w} + d \tag{184}$$

From Darcy Weisbach relationship

$$h_f = Q_i^2 \left[\frac{8f\ell}{\pi^2 g d_o^5}\right] \tag{185}$$

where $Q_i$ = flow rate of feed solution into reactor

$f$, friction factor = $\phi$ (Reynolds number)

$d_o$ = inside diameter of Tube B

From Figure 15A

$$z' - d = z \tag{186}$$

From Equations 182, 183, 184, 185, and 186

$$Q_i = \frac{\pi d_o^2}{2} \left[\frac{g d_o}{2f\ell}\right]^{-\frac{1}{2}} (d_a - z)^{\frac{1}{2}} \tag{187}$$

Since $$\frac{\pi d_o^2}{2} \left[\frac{g d_o}{2f\ell}\right]^{-\frac{1}{2}} = \text{constant, } K \tag{188}$$

$$Q_i = K(d_a - z)^{\frac{1}{2}} \tag{189}$$

Equation 189 is identical to Equation 126 of Chapter V. From Equation 189, for a fixed depth of Tube A in the Lute and an adjusted elevation of the open end of the Tube B, the flow rate remained constant. Furthermore, for the assumption of the derivation to hold, i.e., for flow to occur, $d_a > z$ for $Q_i$ to be positive in Equation 189. If the free end of Tube B is adjusted at a level such that $d_a < z$, $Q_i$ becomes imaginary from Equation 189 and under this condition no flow could occur.

It follows from the above discussions that the depth of the Lute should be at least one inch more than the maximum lift, z, expected.

Satisfactory working of the constant head device depends very much on the formation and steady release of spherical capped air bubbles from air Tubes A and C. The tubes should be of suitable diameter with square edges at the open ends. The edges of the opening of the air tubes should be free of irregularities. In some instances the level of the meniscus inside Tube C oscillated at extremely low flow rates, thus introducing a simultaneous fluctuation in the flow rate.

After escape of the bubble in the nutrient reservoir, the level of fluid in Tube C rises by a few millimeters thereby reducing z. Unfortunately, the phenomenon is more pronounced in low flow rates associated with small reactor volumes and high detention times. To stabilize z, DeHaan and Winkler (321) have suggested that Tube C be fitted with a horizontal arm or with a horizontal spiral ending in a fine point. Such an attachment was not necessary, however, in the apparatus used in this research, since the reactor volume was comparatively larger, and the phenomenon was not noticeable at detention times below four hours.

# APPENDIX III

## DETAILED PROCEDURE FOR THE DEHYDROGENASE TEST

### Preparation of Reagents

1. Tris-HCl buffer, 0.05M : Add 6.037 grams of tris (hydroxymethyl) aminomethane and 20 milliliters of 1.0 N HCl to one liter of distilled water. Buffer pH is 8.4.

2. TTC-glucose reagent: Dissolve 0.200 gram of triphenyltetrazolium chloride (TTC)[*] and 1.500 grams of glucose in 100 milliliters of distilled water. Store reagent at 2°C to prevent growth. Any reddish tinge is indicative of growth and the reagent must be discarded.

3. Ninety-five percent or absolute ethanol

### Procedure

1. Set up three non-protein nitrogen (NPN) digestion tubes (graduated at 35 and 50 ml marks) and add one milliliter of buffer to each test tube. Mark test tube "B" to indicate reagent blank and mark the other two "S" to contain duplicate aliquots of the sample.

2. Homogenize[**] sample at 15,000 rpm for two minutes and pipette an eight milliliter aliquot into each of the two tubes marked "S". Pipette eight milliliters of distilled water to the tube marked "B".

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[*] Eastman Kodak No. 6533

[**] Waring "Blender", single speed for 115 volts ac with "Semi-micro Monel Metal" container and "Friction-fit" cover, Model 700 A, Waring Products Corporation, New York, New York.

3. Place the tubes in a 37°C water bath, stopper, and connect in series as shown in Figure 22. Bubble nitrogen at the rate of 0.01-0.02 SCFM for 10 minutes to bring sample temperature to 37°C, ensure complete purging of DO, and establish a nitrogen atmosphere inside of the tubes.

4. After 10 minutes, add one milliliter of TTC-glucose reagent to all of the tubes and continue to bubble nitrogen during the next 60 minutes of reaction at 37°C. The tubes should be protected from exposure to strong light during the reaction time (a black sheet of plastic was used to cover all tubes).

5. At the end of 60 minutes, stop color development by adding one milliliter of formaldehyde. Also add one milliliter of 4 M HCl to each tube to prevent formation of a colloidal suspension.

6. Stop nitrogen flow, disconnect NPN tubes from each other, wash the inside and outside of nitrogen supply lines within the NPN tubes with ethanol (conveniently done by a few squirts of ethanol from a squeeze-type plastic wash bottle), and allow the washings to drain into the proper NPN tubes. Dilute the contents of the tubes to the 50 milliliter mark with ethanol. Mix diluted solutions thoroughly. (Higher or lower dilutions may be used if color is too intense or too faint.)

7. Allow the samples to stand in darkness for 30 minutes for dissolution of the tetraformozan in alcohol.

8. After 30 minutes in darkness, mix the contents of the tubes, filter through cotton in a funnel and into matched cuvettes for spectro-

photometric analysis[*] at 483 mμ and 0.05 mm slit width, setting the reagent blank at zero absorbancy. A light path of 10 centimeters was used if the percent transmittance with a one centimeter light path was higher than 90 percent.

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[*] Photometric analysis was performed with a Beckman DU Spectrophotometer, Model 2400 (Beckman Instruments, Inc., California) with either Beckman corex square cells of 10 mm light path or Beckman cylindrical cells of 100 mm light path.

# APPENDIX IV

## MINIMAL MEDIA FOR BATCH CULTURES

| Nutrients | Concentrations of Stock Solutions (gm/ℓ) | Amounts Used per Liter of Culture Volume (mg/ℓ) |
|---|---|---|
| $(NH_4)_2SO_4$ | 44.130 | 5 |
| $CaCl_2$, $2H_2O$ | 0.400 | 5 |
| $FeCl_3$, $6H_2O$ | 0.400 | 5 |
| $MgCl_2$, $6H_2O$ | 32 | 5 |
| $Na_2HPO_4$ | 227.2 | 25 (0.04 M) |
| $KH_2PO_4$ | 217.6 | 25 (0.04 M) |
| Glucose | 0.1 | as selected |
| Galactose | 0.1 | as selected |

# APPENDIX V

## PROCEDURE FOR THE DETERMINATION OF CONCENTRATIONS OF DRY BACTERIAL SOLIDS BY THE CONTROL FILTER GRAVIMETRIC TECHNIQUE

(Adapted from the ASTM "Tentative Methods of Test for Particulate Contaminant in Aviation Fuels," D 2276-65T (408), "Control Filter Procedure Using Standard Monitors" of Millipore Filter Corporation (369) and Aerospace Recommended Practice Arp 785 (370) of the Society of Automotive Engineers, Inc., New York.)

### Equipment and Materials

1. A one-liter vacuum flask with side outlet

2. 30 ml capacity pyrex filter holder (unit comprised of a 300 ml pyrex funnel, "coarse grade" fritted support for funnel, spring-action anodized aluminum holding clamp, and neoprene stopper to fit standard one-liter vacuum flask), Catalog No. XX 1004700, Millipore Corporation, Bedford, Massachusetts

3. Vacuum pressure pump

4. Vacuum hose

5. Forceps with unserrated tips

6. Covered glass petri dishes, 150 mm

7. Drying oven

8. Analytical balance

9. Millipore filters, diameter 47 mm, pore size 0.45 micron, type HA, Millipore Filter Corporation, Bedford, Massachusetts

## Test Procedure

1. Label N pairs of filters with ball-point pen on the filter edge (N is the number of samples to be run). A pair may be marked as 1A and 1B and so on, the numbers denoting the sample numbers in a test series. One of the filters in the pair is the test filter, which will retain the solids, while the other is the control filter. Transfer the pair to a clean and covered petri dish but leave the cover ajar.

2. Allow filters to stand at room temperature and humidity for at least one hour for equilibration to ambient room conditions.

3. Weigh all filters after equilibration and record tare weights. Transfer each pair of filters back to the proper petri dish.

4. Using forceps, place the pair of filters on the fritted base of the filter holder so that the test filter is above the control filter. Place the funnel on top of the fritted base and clamp securely.

5. Pipette the selected volume of sample into the funnel, apply vacuum to the filter, and release vacuum when the fluid level has reached the level of the test filter.

6. Wash the inside of the funnel free of any attached solids by rubbing the glass surface with a plastic spatula while dispensing a steady stream of distilled water; then filter to dryness.

7. Release vacuum, remove clamp and funnel from the holder, and with the forceps carefully remove each filter separately from the fritted base and return it to the proper covered petri dish. The wet filters tend to stick to the glass surface of the petri dish after removing them from the filter holders. To remedy this they may be allowed to rest in an inclined fashion between the bottom and the edge of the petri dish for about five minutes for air drying.

8. Place the covered petri dishes with covers ajar in the drying oven at 100°C for exactly 30 minutes.

9. Remove the dishes from the oven and allow filters to cool and equilibrate to ambient room conditions for at least 15 minutes with the covers ajar.

10. Reweigh all filters and record their final weights.

11. Substract tare weight from the final weight of each filter. The result obtained for the test filter gives the uncorrected weight of the sample solids while that for the control filter gives the loss or gain in weight.

12. Apply control filter weight change as a correction factor to each test filter result, substracting this factor when the control filter shows a weight increase or adding the factor when the control filter shows a weight decrease.

13. Compute the dry bacterial solids concentration from the determined weight of solids and the known volume of the sample filtered.

## APPENDIX VI

## SUMMARY OF BATCH RUNS

| Batch | Substrate | Time (hrs) | Time (mins) | Substrate Conc. (mg/ℓ) | Dehydrogenase Activity @ 483 mμ 1 cm cell (o.d.) | Dehydrogenase Activity @ 483 mμ 10 cm cell (o.d.) | Dry Solids Conc. (mg/ℓ) | Remarks |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| #1* | Glucose | 00 | 00 | 136.3 | 0.0132 | | | |
| | | 2 | 31 | 131.7 | 0.0137 | | | |
| | | 4 | 26 | | 0.0162 | | 5.33 | |
| | | 5 | 16 | 119.0 | | | | |
| | | 6 | 09 | 117.2 | | | | |
| | | 7 | 13 | 116.4 | | | | |
| | | 8 | 19 | 104.5 | | | | |
| | | 9 | 08 | 95.5 | 0.0470 | | 20.00 | |
| | | 9 | 52 | 84.1 | | | | |
| | | 10 | 28 | | 0.0364 | | 28.0 | |
| | | 10 | 44 | 73.0 | | | | |
| | | 11 | 11 | 74.3 | 0.0565 | | 37.0 | |
| | | 12 | 04 | 64.7 | 0.0536 | | 33.0 | |
| | | 12 | 54 | 48.5 | | | | |
| | | 13 | 49 | | 0.0977 | | 44.0 | |
| | | 14 | 03 | 35.0 | | | | |
| | | 14 | 56 | | 0.0398 | | 52.0 | |
| | | 15 | 09 | 20.7 | | | | |
| | | 16 | 01 | 6.3 | | | | |
| | | 16 | 30 | | 0.0659 | | 55.0 | |
| | | 16 | 56 | 0.06 | | | | |

*approximate yield coefficient for glucose = 0.40

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| #2 | Glucose | 00 | 00 | ÷ 200.0 | 0.0128 | | 5.0 | |
| | | 01 | 39 | | 0.0160 | | 6.5 | |
| | | 5 | 44 | | 0.0264 | | 9.0 | |
| #3 | Glucose | 00 | 00 | | 0.0789 | | 47.5 | |
| | | 2 | 00 | | 0.0942 | | 61.0 | |
| | | 3 | 38 | | 0.1934 | | 82.0 | |
| #4 | Glucose | 00 | 00 | | 0.0327 | | 18.0 | |
| | | 1 | 00 | | 0.0388 | | 22.0 | |
| | | 4 | 35 | | 0.1388 | | 68.0 | |
| #5 | Glucose | 00 | 00 | | -- | | 9.5 | |
| | | 1 | 13 | | -- | | 8.5 | |
| | | 2 | 25 | | 0.0069 | | 10.5 | |
| | | 3 | 46 | | 0.0258 | | 24.7 | |
| | | 5 | 24 | | 0.0633 | | 38.7 | |
| #6 | Glucose | 00 | 00 | ÷ 200.0 | 0.0044 | | 1.0 | |
| | | 1 | 05 | | 0.0026 | | 4.0 | |
| | | 2 | 00 | | 0.0072 | | 6.5 | |
| | | 4 | 06 | | 0.0174 | | 10.7 | |
| | | 6 | 33 | | 0.0258 | | 20.7 | |
| | | 9 | 45 | | 0.1173 | | 64.0 | |
| | | 10 | 43 | | 0.2048 | | 110.0 | |
| | | 11 | 04 | | 0.1581 | | 90.0 | Approx 45 min after lowest pt of $O_2$ sag |
| #7 | Glucose | 00 | 00 | ÷ 300.0 | | 0.0050 | 1.0 | |
| | | 11 | 02 | | | 0.1201 | 13.5 | |
| | | 12 | 46 | | 0.0593 | | 36.0 | |
| | | 15 | 08 | | 0.1773 | | 88.0 | |
| | | 16 | 12 | | 0.2427 | | 138.4 | |
| | | 17 | 11 | | 0.2573 | | 162.0 | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| #7 | Glucose | 18 | 04 | | 0.2091 | | 146.0 | |
| | | 18 | 56 | | 0.2226 | | 132.0 | |
| | | 20 | 57 | | 0.2253 | | 122.0 | |
| | | 23 | 01 | | 0.3528 | | 126.0 | Lowest pt of oxygen sag |
| | | 23 | 01 | | | 0.2262 | 25.2 | 20% sample + 80% $H_2O$ |
| | | 23 | 01 | | | 0.1297 | 12.6 | 10% sample + 90% $H_2O$ |
| | | 23 | 01 | | | 0.0655 | 6.3 | 5% sample + 95% $H_2O$ |
| #1 | Galactose | 00 | 00 | | 0.0171 | | 8.5 | |
| | | 00 | 56 | | 0.0198 | | 6.0 | |
| | | 1 | 56 | | 0.0237 | | 10.0 | |
| | | 2 | 56 | | 0.0223 | | 12.0 | |
| | | 3 | 56 | | 0.0315 | | 13.0 | |
| | | 4 | 56 | | 0.0463 | | 22.0 | |
| | | 6 | 00 | | 0.0872 | | 36.0 | |
| | | 6 | 58 | | 0.0932 | | 47.0 | |
| | | 8 | 06 | | -- | | 73.0 | Lowest pt of $O_2$ sag |
| | | 8 | 06 | | 0.0738 | | 29.2 | 40% sample + 60% $H_2O$ |
| | | 8 | 06 | | 0.0487 | | 14.6 | 20% sample + 80% $H_2O$ |
| | | 8 | 06 | | 0.0115 | | 3.65 | 5% sample + 95% $H_2O$ |
| #2 | Galactose | 1 | 05 | | 0.0126 | | 5.0 | |
| | | 2 | 34 | | 0.0141 | | 9.5 | |
| | | 3 | 38 | | 0.0155 | | 12.0 | |
| | | 5 | 14 | | 0.0214 | | 40.1 | |
| | | 6 | 41 | | 0.0600 | | 33.0 | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| #3 | Galactose | 00 | 00 | ÷ 200.0 | 0.0022 | | 3.5 | |
| | | 01 | 25 | | 0.0032 | | 9.5 | |
| | | 3 | 05 | | 0.0110 | | 5.0 | |
| | | 4 | 08 | | 0.0066 | | 7.5 | |
| | | 5 | 31 | | 0.0112 | | 18.0 | |
| | | 6 | 32 | | 0.0251 | | 25.3 | |
| | | 7 | 49 | | 0.0182 | | 35.0 | |
| | | 8 | 43 | | 0.489 | | 60.0 | |
| | | 9 | 52 | | 0.0831 | | 56.8 | |
| | | 11 | 02 | | 0.1106 | | 104.0 | Lowest pt of $O_2$ sag |
| | | 13 | 02 | | 0.0429 | | 120.0 | |
| #4 | Galactose | 00 | 00 | | | 0.0685 | 7.0 | |
| | | 01 | 25 | | | 0.0492 | 6.0 | |
| | | 03 | 15 | | | 0.0000 | 10.0 | |
| | | 06 | 48 | | | 0.1276 | 31.0 | |
| | | 08 | 29 | | | 0.2259 | 32.5 | |
| | | 09 | 14 | | | 0.3472 | 36.0 | |
| | | 10 | 04 | | | 0.3780 | 37.0 | |
| | | 10 | 50 | | | 0.3360 | 55.0 | |
| | | 11 | 59 | | 0.0865 | | 82.0 | |
| | | 13 | 02 | | 0.1144 | | 98.0 | |
| | | 15 | 02 | | 0.1515 | | 108.0 | |
| | | 15 | 32 | | 0.1226 | | 116.0 | |
| | | 16 | 14 | | 0.1739 | | 118.0 | |
| | | 17 | 18 | | 0.2417 | | 133.3 | Lowest pt of $O_2$ sag |
| | | 17 | 18 | | 0.1789 | | 106.7 | 80% sample + 20% $H_2O$ |
| | | 17 | 18 | | 0.1033 | | 80.0 | 60% sample + 40% $H_2O$ |
| | | 17 | 18 | | 0.0760 | | 53.3 | 40% sample + 60% $H_2O$ |
| | | 17 | 18 | | 0.0232 | | 26.7 | 20% sample + 80% $H_2O$ |
| | | 17 | 18 | | | 0.0964 | 13.3 | 10% sample + 90% $H_2O$ |
| | | 17 | 18 | | | 0.0614 | 6.7 | 5% sample + 95% $H_2O$ |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| #5* | Galactose | 00 | 00 | | | 0.0506 | 1.0 | |
| | | 00 | 34 | 293.5 | | | | |
| | | 2 | 17 | 336.5 | 0.0092 | | 13.0 | |
| | | 6 | 06 | | 0.0119 | | 15.0 | |
| | | 6 | 24 | 295.0 | | | | |
| | | 7 | 01 | | 0.0103 | | 14.0 | |
| | | 8 | 03 | | 0.0244 | | 28.0 | |
| | | 9 | 03 | 258.0 | 0.0320 | | 40.0 | |
| | | 11 | 10 | | 0.0458 | 0.3835 | 44.0 | |
| | | 12 | 12 | 171.0 | 0.0633 | | 46.0 | |
| | | 13 | 02 | 158.0 | | | | |
| | | 13 | 51 | | 0.0705 | | 52.0 | |
| | | 14 | 03 | 130.0 | | | | |
| | | 15 | 09 | 51.0 | | | | |
| | | 15 | 25 | | 0.1403 | | | |
| | | 15 | 50 | 5.0 | | | | |
| | | 16 | 15 | 3.33 | | | | |
| | | 16 | 43 | | 0.2330 | | 118.0 | 15 min after lowest pt of $O_2$ sag |
| | | 16 | 43 | | 0.1902 | | 94.4 | 80% sample + 20% $H_2O$ |
| | | 16 | 43 | | 0.0790 | | 70.8 | 60% sample + 40% $H_2O$ |
| | | 16 | 43 | | 0.0732 | | 47.2 | 40% sample + 60% $H_2O$ |
| | | 16 | 43 | | 0.0420 | | 23.6 | 20% sample + 80% $H_2O$ |
| | | 16 | 43 | | 0.0209 | 0.1555 | 11.8 | 10% sample + 90% $H_2O$ |
| | | 16 | 43 | | | 0.0585 | 5.9 | 5% sample + 95% $H_2O$ |

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[*] approximate yield coefficient for galactose = 0.31

# APPENDIX VII

## CORRELATION BETWEEN ABSORBANCES OF DEHYDROGENASE SOLUTIONS WITH ONE CENTIMETER AND TEN CENTIMETER LIGHT PATHS

| Dehydrogenase Activity @ 483 mμ | | Source of Sample |
|---|---|---|
| Absorbance in 1 cm cell | Absorbance in 10 cm cell | |
| 1 | 2 | 3 |
| 0.0458 | 0.3835 | Batch 5 on galactose |
| 0.0420 | 0.3790 | Dilution of sample from batch 5 on galactose |
| 0.0209 | 0.1555 | " " |
| 0.0746 | 0.5959 | Dilution of sample from batch 7 on glucose |
| 0.1040 | 0.8333 | " " |
| 0.0223 | 0.1981 | Continuous culture on galactose, Run 10 |
| 0.0437 | 0.3765 | " " " Run 10 |
| 0.0246 | 0.2095 | " " " Run 7 |
| 0.0330 | 0.2467 | " " " |
| 0.0346 | 0.1932 | " " " |
| 0.0608 | 0.5387 | " " " |
| 0.0389 | 0.3382 | " " " |
| 0.0256 | 0.2104 | " " " |
| 0.0265 | 0.2104 | " " " |
| 0.0162 | 0.0543 | " " " |
| 0.0159 | 0.0899 | " " " ↓ |
| 0.0126 | 0.1850 | " " " Run 8 |
| 0.0097 | 0.1548 | " " " |
| 0.0115 | 0.1581 | " " " |
| 0.0037 | 0.0878 | " " " |
| 0.0027 | 0.1074 | " " " |
| 0.0020 | 0.0949 | " " " |
| 0.0011 | 0.0840 | " " " |
| 0.0013 | 0.0732 | " " " |
| 0.0035 | 0.0846 | " " " |
| 0.0013 | 0.0568 | " " " ↓ |

| 1 | 2 | 3 |
|---|---|---|
| 0.0223 | 0.2104 | Continuous culture on galactose, Run 4 |
| 0.0135 | 0.1697 | " " " |
| 0.0203 | 0.2237 | " " " |
| 0.0142 | 0.1878 | " " " |
| 0.0205 | 0.2331 | " " " |
| 0.0279 | 0.3863 | " " " |
| 0.0137 | 0.2042 | " " " |
| 0.0173 | 0.1598 | " " " |
| 0.0075 | 0.1004 | " " " |
| 0.0086 | 0.1883 | " " " |
| 0.0141 | 0.1991 | " " " ↓ |
| | | |
| 0.0396 | 0.2904 | Continuous culture on glucose- Run 3 |
| 0.0789 | 0.6295 | " " galactose, Run 3 |
| 0.0377 | 0.2031 | " " " Run 4 |
| 0.0438 | 0.2845 | " " " |
| 0.0311 | 0.2065 | " " " |
| 0.0227 | 0.2703 | " " " |
| 0.0334 | 0.2090 | " " " |
| 0.0304 | 0.2577 | " " " |
| 0.0232 | 0.1683 | " " " ↓ |

## APPENDIX VIII

## ENZYMATIC METHOD OF THE DETERMINATION OF GLUCOSE USING WORTHINGTON GLUCOSTAT

Procedure I: Concentrations below 30 mg/ℓ

A. Preparation of Reagents

1. Glucostat

(a) Dissolve the contents of the Chromogen vial by adding distilled water to the vial with a squeeze bottle. Pour solution into a clean 10 ml graduate cylinder. Rinse vial several times with small amounts of distilled water and add rinses to solution in the cylinder.

(b) Dissolve the contents of a Glucostat vial in a similar manner and add the Glucostat solution to the Chromogen solution. Dilute the mixture to the 50 ml mark of the graduate cylinder, mix and store in darkness in a refrigerator. If the reagent is turbid, it should be filtered through a Whatman No. 30 (or No. 40) filter paper.

4. 4 M HCl

Dilute 340 ml of concentrated HCl (reagent grade) to one liter with distilled water.

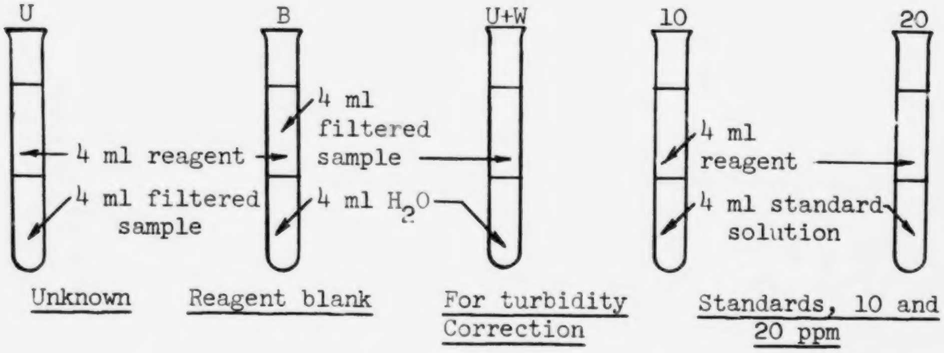
3. Standard Glucose Solutions

(a) Stock solution: Dissolve 2.5 grams of benzoic acid (preservative) in about 900 ml of boiling distilled water. Allow the solution to cool to room temperature and dissolve one gram of dextrose (reagent grade) in the benzoic acid solution and finally dilute the solution to one liter in a volumetric flask. The solution may be stored in a cold place.

(b) Prepare standard glucose solutions of various selected concentrations by proper dilutions of the stock solution.

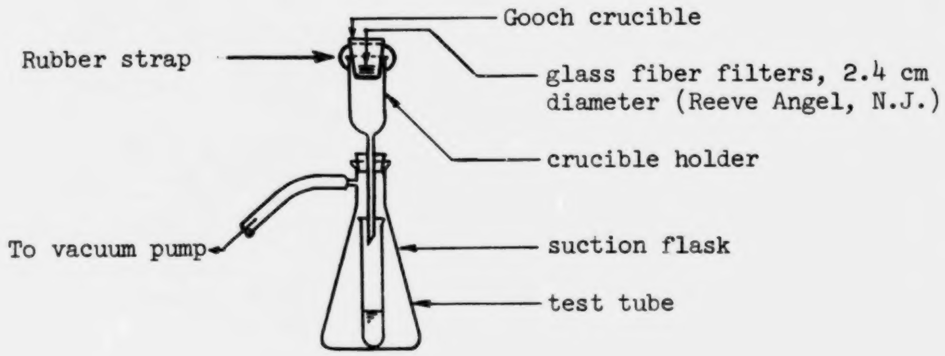
B. Analysis of the Unknown Sample

1. Set up several 18 x 150 mm test tubes labeled as follows:


U
B
U+W
10
20
4 ml reagent
4 ml filtered sample
4 ml filtered sample
4 ml $H_2O$
4 ml reagent
4 ml standard solution
Unknown
Reagent blank
For turbidity Correction
Standards, 10 and 20 ppm

Pipette 4 ml of distilled water into tubes marked "B" and "U+W". Pipette 4 ml of standard solution into tubes appropriately marked with the value of the concentrations. The concentrations of the standard solutions used should approximate the concentration of the unknown. It is preferable to use more than one standard solution as the unknown concentration frequently cannot be guessed. The unknown is run in duplicate.

2. Set up filtration apparatus for filtering sample from the reactor as follows:


Gooch crucible
Rubber strap
glass fiber filters, 2.4 cm diameter (Reeve Angel, N.J.)
crucible holder
To vacuum pump
suction flask
test tube

3. Filter reactor sample by slowly pouring it at the center of the glass filter and avoiding flooding of the filter paper or short circuiting through the edges of the filter paper. Good filtration can be obtained when vacuum reads 20 psig or more.

4. Pipette 4 ml of filtrate into test tube marked "U" and 4 ml into test tube marked "U+W". Add 4 ml of the Glucostat reagent to all the tubes except the tube labeled "U+W". Incubate all tubes at 37°C in a water bath for 30 minutes.

5. After 30 minutes, add two drops of 4 M HCl to each tube to stop the reaction and color development. Mix contents of the tubes by gently tapping the bottom of the tubes. Allow the tubes to stand at room temperature for five minutes. The color remains stable for at least 12 hours.

6. Read the percent transmittance of the contents of Tube "U+W", "B", standards and "U" in the order stated in a spectrophotometer at a wavelength of 400 mμ and a 0.09 mm slit width with respect to a distilled water blank. (Colorimetric analysis was performed with a Beckman DU spectrophotometer, Model 2400.)

7. Compute the concentration of the unknown from the following

formula:

$$C_u = \frac{C_s}{(A_{st}-A_b)} X(A_u-A_b-A_t) \qquad (190)$$

$C_u$ = concentration of unknown

$C_s$ = concentration of standard

$A_{st}$ = 2-log (% T of standard solution)

$A_u$ = 2-log (% T of unknown solution)

$A_b$ = 2-log (% T of reagent blank)

$A_t$ = 2-log (% T due to turbid solution in Tube "U+W")

Procedure II: Concentrations above 30 mg/ℓ

A. Preparation of Reagents

Use same as in Procedure I with the exception that the Glucostat reagent is diluted to 90 ml instead of 50 ml.

B. Analysis of the Unknown Sample

Step 1 is the same as Step 1 of Procedure I, but one ml of distilled water is used in the tubes marked "B" and "U+W" and one ml of standard solution is pipetted in the appropriately labeled test tubes. Steps 2 and 3 are the same as the corresponding steps of Procedure I. In Step 4, one ml of filtrate is pipetted into tubes "U" and "U+W" and 9 ml of glucostat reagent is used instead of the 4 ml used in Procedure I. Steps 5 through 7 are the same as those in Procedure I.

# APPENDIX IX

## ENZYMATIC METHOD OF THE DETERMINATION OF GALACTOSE USING WORTHINGTON GALACTOSTAT

Procedure I: Concentrations below 50 mg/ℓ

A. Preparation of Reagents

1. Galactostat:

(a) Use two vials of Chromogen and two vials of Galactostat. Dissolve contents of the Chromogen vials by adding 0.5 ml of methanol into the vial and mixing. Pour solutions into a 100 ml graduate cylinder. Rinse the vials and vial caps using small amounts of distilled water and add rinses to the cylinder.

(b) Add about 8 ml of distilled water to each Galactostat vial, recap vials, and shake to dissolve contents. Add solutions to the 100 ml cylinder. Rinse Galactostat vials and vial caps with distilled water and pour rinses into the graduate. The solution should be filtered through Whatman No. 30 or No. 40 filter papers if turbidity develops. Dilute the solution in the graduate to 50 ml, mix, and store in a dark bottle in a refrigerator. The reagent should not be freezed to avoid denaturation of the enzyme protein.

2. EDTA Solution:

Prepare a saturated solution of EDTA. Filter and store filtrate in a stoppered bottle.

3. Standard Galactose Solutions:

The procedure is similar to that for the preparation of the glucose standards (see Appendix VIII).

B. Analysis of the Unknown

The procedure involves the same number of steps as in the Glucostat test. The operations are also very similar with the following exceptions:

(a) In Step 1, pipette 2 ml of distilled water into the tubes labeled "B" and "U+W" and 2 ml of standard galactose solution into the tubes for standard solutions.

(b) In Step 4, 2 ml of filtrate is pipetted into the tube marked "U" and another 2 ml into the tube marked "U+W". All tubes excepting the "U+W" receive 2 ml of galactostat reagent. All tubes are incubated at 37°C for at least an hour. Longer incubation periods up to two hours may be needed for very dilute galactose solutions.

(c) In Step 5, the reaction is stopped by adding 0.267 ml of saturated solution of EDTA with a micropipette. The color is not as stable as that of Glucostat.

(d) In Step 6, DU analysis is performed at a wavelength of 425 millimicrons and slit width of 0.09 mm.

Procedure II: Concentrations above 50 mg/ℓ

A. Preparation of Reagents

Same as in Procedure I but use one vial of Galactostat and one vial of Chromogen.

B. Analyses of the Unknown

Same as in Procedure I except that 3 ml of Galactostat reagent is used instead of 2 ml. The incubation period need not be more than one hour as adequate intensity of color is developed at the end of this duration of incubation.

# APPENDIX X

## ESTIMATION OF THE OVERALL OXYGEN TRANSFER COEFFICIENT $K_La$ FOR CONTINUOUS CULTURE

The rate of oxygen consumption by a biological culture is given by

$$\frac{dX_\theta^{O_2}}{d\theta} = p\,\frac{dX_\theta^S}{d\theta} \qquad (191)$$

where $X_\theta^{O_2}$ = concentration of oxygen in culture at anytime, $\theta$

$p$ = mass of oxygen consumed per unit mass of substrate assimilated

From mass balance

$$\frac{dX_\theta^S}{d\theta} = \frac{1}{Y^o}\,\frac{dX_\theta^o}{d\theta} \qquad (192)$$

where $X_\theta^S$ = substrate concentration

$Y^o$ = observed growth yield

$X_\theta^o$ = organism concentration

Also

$$\frac{dX_\theta^o}{d\theta} = kX_\theta^o \qquad (193)$$

where $k$ = specific growth rate ($\text{time}^{-1}$)

Steady state organism concentration in reactor is given by

$$X_1^o = (X_o^s - X_1^s)Y^o \qquad (194)$$

where $X_1^o$ = steady state organism concentration in reactor

$X_o^s$ = influent concentration of substrate

$X_1^s$ = steady state concentration of substrate in reactor

Also,

$$k \approx 1/\theta_r \qquad (195)$$

where $\theta_r$ = detention time in reactor

From Equations 191, 192, 193, 194, and 195

$$\frac{dX_1^{O_2}}{d\theta} = \frac{P(X_o^s - X_1^s)Y^o}{Y^o \theta_r} \qquad (196)$$

where $X_1^{O_2}$ = steady state oxygen concentration in reactor

Let

$$P/Y^o = R \qquad (197)$$

where R = mass of oxygen consumed per unit mass of organism in reactor

Substituting Equation 197 into Equation 196

$$\frac{dX_1^{O_2}}{d\theta} = \frac{R}{\theta_r}(X_o^s - X_1^s)Y^o \qquad (198)$$

The physical oxygen transfer rate is given by

$$\frac{dX_1^{O_2}}{d\theta} = K_L a\,[\alpha X_s^{O_2} - X_1^{O_2}] \qquad (199)$$

where $X_s^{O_2}$ = saturation concentration of oxygen in distilled water

$$\alpha = \frac{K_L a \text{ for culture}}{K_L a \text{ for distilled water}} \qquad (200)$$

As steady state

$$\frac{R}{\theta_r}(X_0^S - X_1^S)Y^0 = K_L a\,(\alpha X_s^{O_2} - X_1^{O_2}) \qquad (201)$$

and

$$K_L a = \frac{R(X_0^S - X_1^S)Y^0}{\theta_r(\alpha X_s^{O_2} - X_1^{O_2})} \qquad (201A)$$

Equation 201A is identical with Equation 147 of Chapter VII.

Assume: (1) $X_1^S = 0$ and $Y^0 = 0.7$ for glucose grown cells at

$\theta_r = 6$ hrs and 30°C

(2) $X_0^S = 290$ mg/ℓ and $Y^0 = 0.8$ for glucose grown cells at

$\theta_r = 1$ hr and 30°C

(3) $X_0^S = 300$ mg/ℓ

(4) $\alpha = 0.95$

$X_s^{O_2} = 7.63$ at 30°C from Standard Methods (327)

$R = 0.77$ at 30°C from Schulze (64,65)

If it is desired to have a residual DO of 2 ppm, then

$$X_1^{O_2} = 2$$

From Equation 201A

For a detention time of 6 hours

$$K_L a = 4.8 \text{ hr}^{-1} \text{ at } 30°C,$$

and for a detention time of 1 hr,

$$K_L a = 1.095 \text{ hr}^{-1} \text{ at } 30°C.$$

Using the usual expression for the effect of temperature on the oxygen transfer coefficient (328) and assuming a temperature coefficient of 1.05, the design $K_La$ values at 20°C were computed to be 3.93/hr and 0.90/hr at detention times of six and one hour, respectively.

Computation of Coefficient, $\alpha$

$K_La$ varies inversely as the volume so that

$$\frac{K_La'}{K_La} = \frac{V}{V'} \tag{202}$$

where $K_La$ and $K_La'$ are transfer coefficients at volumes V and V', respectively

From Figure 34

$K_La = 10.3\ hr^{-1}$ for sterilized culture (5470 ml) and tap water (5112 ml)

From Equation 202

$$K_La \text{ for 5112 ml of sterilized culture} = \frac{10.3\ (5470)}{5112}$$

$$= 11.00\ hr^{-1}$$

$$\text{Coefficient, } \alpha = \frac{K_La \text{ of sterilized culture of volume 5112 ml}}{K_La \text{ of sterilized culture of volume 5112 ml}} = 1.07$$

# APPENDIX XI

## THEORETICAL BASIS FOR GRAPHICAL DETERMINATION OF $K_La$

The mathematical basis for the graphical technique as given below is similar to Fair's (409) method of analysis of BOD data by the method of log difference.

The rate of oxygen absorption is expressed by the equation

$$\frac{dX_\theta^{O_2}}{d\theta} = K_La\ (X_s^{O_2} - X_\theta^{O_2}) \qquad (203)$$

Since the dissolved oxygen deficit

$$D = X_s^{O_2} - X_\theta^{O_2} \qquad (204)$$

Equation 203 reduced to

$$-\frac{dD}{d\theta} = K_LaD \qquad (205)$$

Solution of the differential Equation 205 is

$$D_\theta = D_0 e^{-K_LaD} \qquad (206)$$

where $D_0$ = saturation deficit at $\theta=0$

$D_\theta$ = saturation deficit at $\theta=\theta$

Consider the reoxygenation period to be divided into a number of intervals each being equal to the constant duration, $\Delta\theta$. In other words, if $\theta_0$, $\theta_1$, $\theta_2$, $\theta_3$, etc. are points in time separating the intervals,

then
$$\theta_1-\theta_0 = \theta_2-\theta_1 = \theta_3-\theta_2 = \ldots .\Delta\theta \tag{207}$$

It now follows from Equations 206 and 207 that

$$D_\theta - D_{(\theta+\Delta\theta)} = D_0 e^{-K_L a\theta}\left[1-e^{-(K_L a)(\Delta\theta)}\right] \tag{208}$$

The left hand side of Equation 208 represents the decrease in saturation deficit over the interval $\Delta\theta$ and is the same as the increase in concentration of oxygen $\Delta X^{O_2}_{\Delta\theta}$ during the same interval, so that

$$\Delta X^{O_2}_{\Delta\theta} = D_0 e^{-K_L a\theta}\left[1-e^{-(K_L a)(\Delta\theta)}\right] \tag{209}$$

or

$$\ln \Delta X^{O_2}_{\Delta\theta} = \ln D_0 \left[1-e^{-(K_L a)(\Delta\theta)}\right] - K_L a\theta \tag{210}$$

Equation 210 is identical to Equation 148 of Chapter VII.

Thus, the logarithm of incremental oxygen absorption bears a linear relationship with time which is the basis of the plot of Figure 34. It is to be noted that

$$\Delta X^{O_2}_{\Delta\theta} = X^{O_2}_{\theta_1} - X^{O_2}_{\theta_0} \quad \text{or} = X^{O_2}_{\theta_2} - X^{O_2}_{\theta_1}\ , \text{ etc.} \tag{211}$$

# APPENDIX XII

## DESIGN OF PHOSPHATE BUFFER SYSTEM

Maximum production of $CO_2$ is expected at the higher detention times. For an assumed glucose or galactose consumption of 300 mg/ℓ, a yield coefficient of 0.6 and considering that each mole of glucose yields six moles of carbon dioxide on combustion via the glycolytic and tricarbonylic cycle, the carbon dioxide production may be calculated as:

$$CO_2 \text{ production} = (300)\ (0.4)\ (264)/180 = 176 \text{ mg}/\ell$$
$$= 4 \times 10^{-3} \text{ moles}/\ell$$

It is doubtful that all of the $CO_2$ produced would be in solution and remain as carbonic acid at the pH, alkalinity, and total solids concentrations encountered in the solution of the nutrient salts. Nevertheless, since it was difficult to calculate the concentration of the carbonic acid which would be actually present, it was further assumed that

$$[CO_2] = [H_2CO_3] = 4 \times 10^{-3} \text{ moles}/\ell$$

If the acceptable pH deviation is 0.1 unit, then the design buffer index was

$$\beta = \frac{4 \times 10^{-3}}{0.1} = 4 \times 10^{-2} \text{ moles}/\ell \text{ as } H_2CO_3$$

From Equation 13, page 243 of Butler (393) and using the design $\beta = 4 \times 10^{-2}$ moles/ℓ and pH of the equimolar $H_2PO_4^-$ = $HPO_4^=$ system to be 6.88 at 20°C (392), the total phosphate concentration was calculated to be 0.08 M. The buffer system therefore constitutes a mixture of 0.04 M $KH_2PO_4$ and 0.04 M $Na_2HPO_4$.

The inverse slope or buffer index, β, of the buffer solution used was 66.6 meq/ℓ per pH unit approximately (see Figure 37). Expressed in terms of carbonic acid concentration, the buffer index, β, of the buffer solution used was $3.33 \times 10^{-2}$ moles/ℓ compared to the desired buffer index of $4 \times 10^{-3}$ moles/ℓ.

# APPENDIX XIII

## SAMPLE CALCULATIONS FOR CONCENTRATIONS OF NUTRIENT SALTS

### Determination of Concentration of the Nutrient Salt Furnishing the Nitrogen Requirement

Assume substrate glucose or galactose concentration to be 300 mg/ℓ maximum after dilution.

Carbon concentration in substrate feed = (72) (300)/180 = 120 mg/ℓ

Using $(NH_4)_2SO_4$ as the nutrient salt and a C:N ratio of 6, the concentration of $(NH_4)_2SO_4$ in the influent solution after dilution by substrate flow should be:

$$(NH_4)_2SO_4 \text{ (in mg/}\ell) = \left(\frac{120}{6}\right)\left(\frac{132}{28}\right) = 94.3$$

Considering a maximum dilution factor of 12 due to dilution by the substrate solution,

$$(NH_4)_2SO_4 \text{ (in mg/}\ell) = (94.3)\ (12) = 1131.6$$

This concentration may also be computed from Equation 152 of Chapter VII as follows:

$$M^{ns} = 132,\ W^{ne} = 28,\ X_o^s = 300,\ W^c = 72,\ M^s = 180,\ R = 6,$$
$$\text{and } Q^t/Q^n = 12, \text{ so that,}$$

$$X_f\ (NH_4)_2SO_4 = \frac{132 \times 300 \times 72 \times 12}{28 \times 180 \times 6} = 94.3 \text{ mg/}\ell$$

Concentrations of Nutrient Salts in the Nutrient Feed Reservoir

| Nutrient Salts | Concentrations (mg/ℓ) |
|---|---|
| $(NH_4)_2SO_4$ | 1,290 |
| $Na_2HPO_4$ | 22,000.0 |
| $KH_2PO_4$ | 21,000.0 |
| $MgCl_2$, $6H_2O$ | 448.0 |
| $CaCl_2$, $2H_2O$ | 5.6 |
| $FeCl_3$, $6H_2O$ | 5.6 |

# APPENDIX XIV

## EXPERIMENTAL DATA FROM CONTINUOUS CULTURE RUNS

## Part A. Details of Steady State Runs with Glucose as Substrate

| Run No. | Time (hrs) | Time (min) | Glucose Concentrations Reactor (mg/ℓ) | Glucose Concentrations Influent (mg/ℓ) | Dehydrogenase Activity (Absorbance) | Dry Solids Concentration (mg/ℓ) | Flow Rate (ℓ/hr) | Temp (°C) | DO (mg/ℓ) | pH | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 00 | 00 | 1.3 | 210.3 | 0.5767 | | 1.717 | | | 5.95 | |
| | 23 | 48 | 5.2, 2.9 | 192.0 | | | | 20.0 | | 5.30 | Dehydrogenase activities measured with 10 cm light path |
| | 55 | 23 | 4.4 | | | | | | | | |
| | 58 | 09 | | | | | 1.580 | 21.0 | | | |
| | 63 | 08 | | | 0.4894 | | | | | | |
| | 63 | 50 | | | | | | | | | |
| | 65 | 23 | 1.2, 0.6 | 209.5 | | | | | | 6.80 | |
| | 68 | 12 | | | | | | 20.9 | | 6.80 | |
| | 68 | 55 | | 208.0 | | | | | | | |
| | 69 | 02 | 2.0 | | 0.4969 | | | | | | |
| | 69 | 58 | | | | | 1.560 | 20.8 | | 6.80 | |
| | 78 | 28 | | | | | | 20.2 | | 6.80 | |
| | 78 | 53 | | 209.0 | | | | | | | |
| | 79 | 00 | 6.8, 4.1 | | | | | | | | |
| | 80 | 13 | | | | | 1.560 | 20.3 | | | |
| | 80 | 43 | | | | | 1.620 | | | | |
| | 95 | 43 | 0.0 | 185.3 | 0.5823 | | | 19.0 | | 5.35 | |
| 1 | 114 | 03 | | | | | | 20.3 | | 6.66 | |
| | 120 | 33 | 0.0 | | 0.5500 | | | | | 6.60 | |
| | 120 | 58 | | | | | | | | | |
| | 143 | 43 | 0.0 | 215.5 | | | | | | | |
| | 147 | 53 | | | | | 1.410 | 20.5 | | 6.70 | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 149 | 13 |  |  |  |  |  |  |  |  |  |
|  | 160 | 28 | 5.4 |  | 0.5098 |  |  |  |  |  |  |
|  | 160 | 58 |  |  |  |  | 1.340 | 20.5 |  | 6.76 |  |
|  | 161 | 23 |  |  |  |  | 1.130 |  |  |  |  |
|  | 167 | 43 | 0.0 | 211.0 |  |  |  | 20.0 |  | 6.76 |  |
|  | 184 | 09 |  |  |  |  | 1.080 |  |  |  |  |
|  | 185 | 28 | 4.5 |  |  |  |  |  |  |  |  |
|  | 191 | 08 | 0.0 |  | 0.5473 |  |  | 20.0 |  | 6.60 |  |
|  | 209 | 13 |  |  | 0.5430 |  | 1.488 | 20.5 |  | 6.65 |  |
|  | 210 | 28 |  |  |  |  |  |  |  |  |  |
|  | 223 | 43 |  |  |  | 86.0 |  | 20.3 |  | 6.75 |  |
|  | 223 | 48 | 2.0 | 182.7 | 0.5064 |  |  |  |  |  |  |
|  | 224 | 43 |  |  |  |  |  |  |  |  |  |
|  | 225 | 58 |  |  |  |  | 1.292 |  |  |  |  |
|  | 226 | 13 |  |  |  |  | 1.327 |  |  |  |  |
|  | 226 | 43 |  |  | 0.6284 | 92.0 |  |  |  |  |  |
|  | 252 | 03 |  |  | 0.5180 |  | 1.486 | 20.3 |  | 6.70 |  |
|  | 252 | 48 |  |  |  |  | 1.435 |  |  |  |  |
|  | 252 | 56 |  |  |  |  | 1.460 |  |  |  |  |
|  | 255 | 16 | 4.1, 3.9 | 146.0 |  |  |  |  |  |  |  |
|  | 255 | 28 |  |  |  |  |  |  |  |  |  |
|  | 263 | 13 |  |  |  |  |  | 20.5 |  |  |  |
|  | 265 | 33 | 0.0 | 152.0 | 0.6839 |  |  |  |  |  |  |
|  | 270 | 43 |  |  |  | 98.0 | 1.500 |  |  | 6.65 |  |
|  | 271 | 43 |  |  |  |  | 1.330 |  |  |  |  |
|  | 287 | 18 | 6.7 | 167.0 |  |  |  | 21.0 |  | 6.78 |  |
|  | 297 | 02 | 2.5 |  |  | 82.0 |  |  |  |  |  |
|  | 298 | 39 | 3.7 |  | 0.8133 |  |  |  |  |  |  |
| 1 | 299 | 18 |  |  |  | 90.0 | 1.386 | 21.0 |  | 6.75 |  |
|  | 302 | 43 | 3.3 |  |  |  |  |  |  |  |  |
|  | 305 | 43 |  | 142.0 | 0.7372 |  | 1.432 | 20.4 |  |  |  |
|  | 311 | 28 | 4.3 |  |  |  |  | 20.0 |  | 6.70 |  |
|  | 316 | 53 |  |  |  | 98.0 | 1.380 |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 317 | 43 | 4.4 | | | | | | | | |
| | 323 | 20 | 3.0 | 141.6 | | | 1.386 | | | | |
| Steady State | | | 2.8 | 184.7 | 0.5845 | 91.0 | 1.410 | 20.4 | 6.2 | 6.54 | Steady State DO obtained from recorder |
| | 00 | 00 | | | | | | 20.0 | | | Dehydrogenase Activities measured with 10 cm light path |
| | 00 | 45 | | | | | 1.855 | | | 6.75 | |
| | 1 | 10 | 4.2 | | 0.3138 | 101.0 | 1.820 | | | | |
| | 1 | 43 | | | | | | | | | |
| | 2 | 15 | | | | | 1.880 | | | | |
| | 3 | 20 | 6.1 | | | | | | | | |
| | 6 | 30 | | | 0.3601 | | | 20.5 | | 6.75 | |
| | 9 | 30 | 2.9 | | 0.3235 | | | 21.0 | | | |
| 2 | 16 | 15 | 1.6 | | | | | | | | |
| | 16 | 30 | | | 0.4754 | 77.0 | 1.970 | 20.4 | | | |
| | 24 | 47 | 2.7 | | | | | | | | |
| | 25 | 18 | | | 0.4554 | 84.0 | 1.840 | | | | |
| | 25 | 30 | | 107.6 | | | 1.850 | 20.0 | | | |
| | 30 | 00 | 1.1 | 153.0 | 0.4289 | | | 20.0 | | 6.70 | |
| | 41 | 28 | 6.7 | | | | | | | | |
| | 41 | 55 | | | 0.3378 | 83.0 | 1.826 | 20.5 | | 6.70 | |
| | 46 | 31 | | | | 83.0 | | | | | |
| | 49 | 26 | 1.8 | | | | | | | | |
| | 50 | 15 | | 137.5 | | | 1.836 | | | | |
| | 62 | 40 | | | | | | | | | |
| 2 | 70 | 31 | 5.3 | | 0.4591 | | 1.760 | 20.7 | | 6.80 | |
| | 73 | 25 | | 143.3 | | | 1.870 | | | | |
| | 93 | 40 | 1.4 | | 0.4169 | 72.0 | 1.830 | 20.6 | | 6.86 | |
| | 97 | 20 | 3.5 | | | | | | | | |
| | 101 | 40 | 1.2 | 134.0 | 0.4409 | | | 20.0 | | | |
| | 117 | 25 | 3.8 | | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 119 | 35 |  |  |  | 73.0 | 1.764 | 20.5 |  |  |  |
|  | 126 | 05 |  | 133.0 | 0.5244 |  |  | 20.0 |  | 6.80 |  |
|  | 133 | 36 | 1.4 |  |  |  |  |  |  |  |  |
|  | 134 | 50 |  |  |  |  |  | 20.5 |  |  |  |
|  | 141 | 50 | 2.4 | 134.0 | 0.4882 |  |  | 20.5 |  | 6.80 |  |
|  | 154 | 00 |  |  | 0.4220 | 79.0 |  | 20.9 |  |  |  |
|  | 157 | 00 |  | 128.9 |  |  |  |  |  |  |  |
|  | 159 | 24 |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 3.1 | 135.1 | 0.4568 | 75.3 | 1.840 | 20.4 | 4.6 | 6.77 | Steady State DO Obtained from recorder |
|  | 0 | 00 | 3.1 |  |  |  |  |  |  |  | Dehydrogenase Activities measured with 10 cm light path |
|  | 0 | 19 |  |  | 0.4802 |  |  |  |  |  |  |
|  | 1 | 00 |  |  |  | 82.0 | 2.49 |  |  | 6.85 |  |
|  | 1 | 16 | 2.8 |  | 0.5086 |  |  |  |  |  |  |
|  | 3 | 11 | 2.5 |  |  |  |  |  |  |  |  |
|  | 3 | 43 |  |  |  | 80.0 |  |  |  |  |  |
|  | 3 | 58 |  |  | 0.4437 |  | 2.54 | 21.4 |  |  |  |
|  | 4 | 06 |  |  |  |  |  |  |  |  |  |
|  | 6 | 56 | 2.9 | 163.5 |  | 84.0 |  |  |  |  |  |
| 3 | 9 | 46 | 2.0 | 152.5 | 0.4318 |  |  | 21.4 |  | 6.80 |  |
|  | 17 | 05 |  |  | 0.3872 |  |  |  |  |  |  |
|  | 17 | 17 | 3.8 |  | 0.4134 |  |  |  |  |  |  |
|  | 22 | 26 |  |  | 0.4330 |  |  |  |  |  |  |
|  | 22 | 24 |  |  |  | 87.5 |  |  |  |  |  |
|  | 23 | 01 |  |  |  |  |  |  |  |  |  |
|  | 24 | 36 |  |  | 0.4841 |  | 2.45 | 21.0 |  | 6.80 |  |
|  | 24 | 51 |  | 154.4 |  | 70.0 | 2.62 |  |  |  |  |
|  | 30 | 11 | 2.1 | 150.0 | 0.4413 |  |  |  |  | 6.85 |  |
|  | 42 | 31 | 0.3 |  |  | 92.0 | 2.59 | 21.0 |  | 6.80 |  |
|  | 47 | 21 |  | 147.2 |  |  | 2.55 |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 60 | 16 |  |  |  | 92.0 | 1.960 | 21.2 |  | 6.86 |  |
| 3 | 60 | 46 | 4.3 | 147.8 |  |  |  |  |  |  |  |
|  | 62 | 46 |  |  | 0.4260 |  | 2.610 | 21.0 |  | 6.80 |  |
|  | 63 | 54 | 1.8 |  |  | 88.0 | 2.740 |  |  |  |  |
| Steady State |  |  | 2.5 | 150.4 | 0.4490 | 84.4 | 2.550 | 21.2 | 3.1 | 6.82 | Steady State DO obtained from recorder |
|  | 00 | 00 | 10.9 |  |  |  | 3.20 |  |  |  |  |
|  | 02 | 10 |  |  |  |  |  |  |  |  |  |
|  | 3 | 45 | 39.7 |  |  |  |  |  |  |  |  |
|  | 3 | 59 |  | 141.7 |  |  | 3.10 | 21.2 |  |  |  |
|  | 6 | 20 | 39.2 |  | 0.0915 | 54.0 |  |  |  |  |  |
|  | 9 | 10 | 55.2 |  | 0.0778 |  |  |  | 5.7 |  |  |
|  | 13 | 20 |  | 166.0 | 0.0706 |  |  | 21.0 | 5.7 | 6.85 |  |
|  | 26 | 29 |  |  |  |  | 3.24 | 19.3 |  |  |  |
|  | 28 | 02 |  |  |  |  | 2.96 |  |  |  |  |
|  | 29 | 48 | 0.2 |  |  |  |  |  |  |  |  |
|  | 32 | 13 | 19.8 |  |  |  |  |  |  |  |  |
|  | 33 | 05 |  |  |  |  | 2.61 | 19.0 | 7.2 |  |  |
|  | 33 | 10 |  | 155.5 |  |  |  |  |  |  |  |
| 4 | 37 | 10 | 13.1 | 147.0 | 0.1146 |  |  | 19.0 |  | 6.85 | Runs 4 & 5 run at the same detention time but at different influent glucose concentration |
|  | 43 | 50 |  |  |  |  |  |  |  |  |  |
|  | 46 | 12 | 6.7 |  | 0.1367 |  |  |  |  |  |  |
|  | 46 | 45 |  |  | 0.1487 | 78.0 |  |  |  |  |  |
|  | 47 | 12 | 10.4 |  | 0.1192 |  |  |  |  |  |  |
|  | 47 | 38 |  |  | 0.1107 |  | 2.89 | 19.5 |  |  |  |
|  | 47 | 40 |  | 155.0 |  |  |  |  |  |  |  |
|  | 55 | 20 | 7.6 |  | 0.1314 | 76.0 |  |  |  |  |  |
|  | 55 | 55 |  | 144.6 |  |  | 3.03 | 20.0 |  |  |  |
|  | 57 | 10 | 0.3 |  |  |  |  |  |  |  |  |
|  | 70 | 35 | 0.7 |  |  | 78.0 |  |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 72 | 05 |  |  |  |  | 2.86 | 20.9 |  |  |  |
|  | 72 | 27 |  |  |  |  |  |  |  |  |  |
|  | 72 | 36 | 0.1 |  |  |  |  |  |  |  |  |
|  | 73 | 02 |  | 152.7 |  |  | 2.99 |  |  |  |  |
|  | 77 | 08 | 3.7 |  | 0.1238 |  |  |  |  |  |  |
|  | 78 | 07 |  |  |  |  | 2.99 |  |  | 6.76 |  |
|  | 79 | 40 | 0.9 |  |  | 98.0 | 3.06 |  |  |  |  |
|  | 80 | 40 |  |  |  |  |  |  |  |  |  |
|  | 81 | 06 | 4.0 |  |  |  |  |  |  |  |  |
|  | 81 | 40 |  | 144.0 |  |  |  |  |  |  |  |
|  | 81 | 41 |  |  |  |  | 3.01 |  |  |  |  |
|  | 81 | 55 |  |  |  |  |  | 20.4 |  |  |  |
|  | 93 | 36 |  |  |  |  |  |  |  |  |  |
|  | 93 | 59 |  |  |  | 101.0 |  |  |  |  |  |
|  | 103 | 36 | 6.9 |  |  |  |  |  |  |  |  |
|  | 104 | 35 |  |  |  |  | 2.90 | 19.6 |  |  |  |
|  | 104 | 45 |  |  |  |  |  |  |  |  |  |
| 4 | 104 | 50 | 5.2 |  | 0.1325 |  |  |  |  |  |  |
|  | 105 | 05 |  | 146.5 |  |  |  |  |  |  |  |
|  | 118 | 20 | 1.4 |  |  |  |  |  |  |  |  |
|  | 119 | 28 |  |  |  |  | 3.01 | 20.5 |  |  |  |
|  | 119 | 45 |  |  |  |  | 2.93 |  |  |  |  |
|  | 121 | 02 |  |  |  |  |  |  |  |  |  |
|  | 138 | 42 | 7.4 | 145.0 | 0.1085 | 70.0 |  |  |  |  |  |
|  | 138 | 53 |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 5.8 | 148.0 | 0.1251 | 85.0 | 2.99 | 20.0 | 6.2 | 6.82 |  |
|  | 140 | 42 | 8.8 |  | 0.0809 | 38.0 |  |  |  |  | Dehydrogenase activities measured with 1 |
|  | 140 | 54 |  |  | 0.0610 | 32.0 |  |  |  |  |  |
|  | 141 | 30 |  | 77.9 |  |  | 2.99 | 21.0 |  |  |  |
|  | 141 | 50 |  |  |  |  | 3.07 |  |  |  |  |
|  | 142 | 28 |  |  |  |  |  |  | 5.6 | 6.77 |  |
|  | 142 | 57 | 4.6 |  |  |  |  |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 144 | 56 |  |  | 0.0645 | 42.7 |  |  |  |  | cm light path. Detention time of Run 5 same as that of Run 4, but influent glucose concentration is about half of that used in Run 4. |
|  | 145 | 10 | 2.6 |  | 0.0830 | 34.7 |  |  |  |  |  |
| 5 | 147 | 35 | 0.8 |  |  | 41.3 |  |  |  |  |  |
|  | 148 | 12 |  | 77.9 |  |  | 3.10 | 21.0 |  |  |  |
|  | 149 | 15 | 0.9 |  | 0.0937 |  |  |  |  |  |  |
|  | 149 | 55 |  |  | 0.0610 | 40.0 |  |  |  |  |  |
|  | 150 | 25 |  | 77.9 |  | 42.7 | 3.01 |  |  |  |  |
|  | 151 | 35 |  |  |  |  | 3.05 |  |  |  |  |
|  | 151 | 40 | 0.6 |  |  |  |  |  |  |  |  |
|  | 151 | 50 |  |  |  |  |  |  |  |  |  |
|  | 163 | 50 |  |  |  |  | 2.90 |  |  |  |  |
|  | 166 | 25 | 1.8 |  | 0.0872 |  |  |  |  |  |  |
|  | 166 | 50 |  | 74.4 | 0.0555 | 41.0 | 2.87 |  |  |  |  |
|  | 169 | 17 | 5.5 |  |  | 37.0 |  |  |  |  |  |
| Steady State |  |  | 5.8 | 77.0 | 0.0734 | 39.1 | 2.99 | 21.0 | 5.6 | 6.77 |  |
|  | 00 | 00 |  |  |  |  |  |  |  |  | Dehydrogenase activities measured with 1 cm light path. |
|  | 1 | 02 | 31.2 |  |  |  |  |  |  |  |  |
|  | 1 | 40 |  |  | 0.1098 |  |  |  |  |  |  |
|  | 1 | 59 |  |  |  | 66.0 |  |  |  |  |  |
|  | 3 | 05 |  | 147.5 |  |  | 3.36 |  |  |  |  |
|  | 3 | 25 | 26.8 |  |  |  |  | 22.0 |  | 6.90 |  |
|  | 4 | 25 |  |  | 0.0812 |  |  | 22.0 |  |  |  |
|  | 5 | 03 |  |  |  |  |  |  |  |  |  |
|  | 6 | 50 |  |  |  |  |  |  |  |  |  |
|  | 7 | 05 |  | 142.5 |  |  | 3.34 |  |  |  |  |
|  | 7 | 25 |  |  |  |  |  | 22.0 |  |  |  |
|  | 9 | 42 |  |  |  |  |  |  |  |  |  |
|  | 10 | 05 |  |  |  |  |  |  |  |  |  |
|  | 11 | 07 |  |  |  |  |  |  |  |  |  |
|  | 11 | 15 |  | 144.8 |  |  |  |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 13 | 13 | | | | | | | | | |
| | 13 | 40 | | | | 56.0 | | | | | |
| | 14 | 40 | | | | | | | | | |
| | 19 | 06 | | | 0.1014 | 72.0 | | | | | |
| | 19 | 33 | | 135.8 | | | 3.39 | 22.7 | | | |
| | 20 | 36 | | | | | | | | | |
| | 22 | 33 | | | | | | | | | |
| | 23 | 23 | | 138.0 | | | 3.43 | 22.8 | | 6.85 | |
| | 30 | 55 | | 147.5 | | | | | | | |
| | 44 | 15 | | | | | | 20.5 | | | |
| | 44 | 40 | | 133.1 | | | 3.22 | 20.3 | | | |
| | 54 | 20 | | 139.0 | 0.0942 | | | 20.0 | 5.0 | 6.85 | |
| | 63 | 30 | | 140.2 | | | 3.32 | 20.1 | | | |
| | 65 | 15 | | | 0.1171 | | | | | | |
| | 81 | 56 | 34.0 | | 0.0443 | 68.0 | | 19.8 | | | |
| | 82 | 35 | | | | | | | | | |
| | 85 | 12 | | | 0.1123 | | | | | | |
| | 86 | 00 | | 132.9 | | | 3.27 | 19.2 | | | |
| | 86 | 30 | | | | | | | | | |
| | 87 | 32 | 26.9 | | 0.1457 | 64.0 | | | | | |
| | 90 | 10 | 34.7 | | 0.1407 | | | | | | |
| | 92 | 20 | 40.0 | | 0.0592 | 64.0 | | | | | |
| | 93 | 00 | | 143.2 | | | 3.31 | | | | |
| | 94 | 10 | 45.0 | | | | | | | | |
| | 102 | 10 | 45.7 | | 0.0755 | 72.0 | | | | 6.75 | |
| | 109 | 48 | 50.5 | | 0.0922 | | | | | | |
| | 113 | 00 | 40.7 | | 0.1167 | 62.0 | | | | | |
| | 116 | 00 | 46.8 | | | | | | | | |
| | 117 | 04 | | | 0.1283 | 64.0 | | | | | |
| | 118 | 35 | | 141.8 | | | | | | | |
| | 118 | 40 | | | | | 3.15 | | 5.8 | | |
| | 119 | 10 | 41.0 | | | 63.0 | | | | | |
| | 132 | 53 | | 150.0 | | | | 18.0 | | | |
| | 133 | 35 | | | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 133 | 48 | 42.6 |  |  | 64.0 |  |  |  |  |  |
| 6 | 134 | 15 |  |  | 0.0758 |  |  |  |  |  |  |
|  | 143 | 40 | 48.0 |  | 0.0633 |  |  |  |  |  |  |
|  | 145 | 40 |  |  |  |  | 3.16 |  |  |  |  |
|  | 158 | 45 |  |  |  |  |  | 18.6 |  |  |  |
|  | 175 | 25 | 33.6 | 166.0 | 0.0870 |  |  | 19.0 |  | 6.70 |  |
|  | 182 | 55 |  | 155.0 |  |  | 3.18 | 22.5 |  |  |  |
|  | 186 | 42 |  |  |  | 56.0 |  | 22.5 |  |  |  |
|  | 190 | 43 | 31.5 |  |  |  |  | 22.0 |  |  |  |
|  | 198 | 20 | 31.0 | 151.9 | 0.0711 |  |  | 20.9 |  | 6.70 |  |
|  | 204 | 37 |  |  |  | 76.0 |  | 20.9 |  |  |  |
|  | 215 | 03 |  |  |  |  |  | 20.9 |  |  |  |
|  | 223 | 00 |  | 136.0 | 0.0869 |  |  | 20.7 |  | 6.75 |  |
|  | 227 | 25 |  |  |  |  |  | 21.0 |  |  |  |
|  | 232 | 15 |  |  |  |  |  |  |  |  |  |
|  | 238 | 54 | 33.7 |  | 0.0658 |  |  |  |  |  |  |
|  | 240 | 40 | 33.1 |  | 0.0605 |  |  |  |  |  |  |
|  | 241 | 55 |  | 156.0 |  |  | 3.29 | 20.5 |  |  |  |
|  | 242 | 23 | 34.2 |  | 0.0549 |  |  |  |  |  |  |
|  | 243 | 35 | 39.5 |  | 0.0543 |  |  |  |  |  |  |
|  | 244 | 53 |  | 151.0 |  |  |  | 20.3 | 2.4 |  |  |
|  | 245 | 30 | 46.5 |  | 0.0524 |  |  |  |  |  |  |
|  | 246 | 35 | 46.5 | 166.0 | 0.0519 |  |  |  |  | 6.75 |  |
|  | 247 | 50 | 47.0 |  | 0.0398 | 62.0 |  | 20.7 |  |  |  |
|  | 253 | 20 | 53.9 |  | 0.0413 |  |  | 21.0 |  |  |  |
|  | 254 | 38 |  | 145.5 |  |  | 3.30 | 20.7 |  |  |  |
| 6 | 263 | 19 |  |  | 0.0445 |  |  |  |  |  |  |
|  | 263 | 47 | 63.0 |  |  |  |  |  |  |  |  |
|  | 265 | 00 |  | 153.5 |  |  | 3.40 | 20.6 |  |  |  |
|  | 266 | 14 | 59.4 |  |  |  |  |  | 6.0 |  |  |
|  | 266 | 23 |  | 141.0 |  |  | 3.30 | 20.7 |  |  |  |
|  | 270 | 15 | 50.5 | 156.5 | 0.0492 | 64.0 |  | 20.5 |  | 6.75 |  |
|  | 278 | 59 | 45.0 |  | 0.1109 |  |  | 20.5 |  |  |  |
|  | 279 | 35 |  | 137.5 |  |  |  | 20.1 |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 282 | 25 | | 128.5 | | | 3.32 | 20.5 | | | |
| | 287 | 28 | 57.2 | | 0.0849 | | | | | | |
| | 300 | 33 | 37.0 | | 0.1209 | | | | | | |
| | 301 | 15 | | 142.5 | | | 3.28 | 20.9 | | | |
| | 301 | 25 | | | | | | | | | |
| Steady State | | | 41.1 | 144.3 | 0.0813 | 64.9 | 3.30 | 20.8 | 4.7 | 6.73 | |
| | 00 | 59 | 55.6 | | 0.1412 | | | | | | Dehydrogenase activities measured with 1 cm light path. |
| | 3 | 51 | 77.3 | | 0.0453 | | | | | | |
| | 8 | 20 | 71.2 | | 0.1035 | | | | | | |
| | 11 | 15 | | 165.0 | | | | | | | |
| | 12 | 20 | 52.0 | | | | | | | | |
| | 13 | 47 | 45.2 | | 0.1403 | | | 21.1 | | 6.75 | |
| | 14 | 10 | | 148.0 | | | | | | | |
| 7 | 15 | 20 | 40.2 | | | | 3.75 | | | | |
| | 16 | 40 | | 144.0 | | | 3.68 | 20.3 | | | |
| | 32 | 25 | | | | | 3.63 | 19.4 | | | |
| | 32 | 40 | | 164.5 | | | | | | 6.80 | |
| | 35 | 40 | | | | | 3.73 | | | | |
| | 40 | 50 | 56.3 | 171.0 | 0.0651 | | | 19.5 | | 6.80 | |
| | 64 | 45 | 63.6 | 176.3 | | | | 19.5 | 1.0 | 6.70 | |
| | 72 | 27 | 65.4 | | 0.1155 | | | 19.2 | | | |
| | 73 | 04 | | 172.5 | | | 3.57 | 18.0 | 7.2 | | |
| | 75 | 00 | 63.0 | | 0.1419 | | | 19.0 | | | |
| | 75 | 36 | | | | | 3.58 | 19.0 | | | |
| | 76 | 34 | 58.8 | | 0.1374 | | | 19.2 | | | |
| | 78 | 27 | 66.3 | | 0.1263 | | | 19.0 | 7.0 | | |
| | 84 | 30 | 65.5 | | 0.0745 | | | | | | |
| | 84 | 45 | | 167.0 | | | 3.45 | 18.6 | | 6.75 | |
| | 89 | 15 | | 176.0 | | 70.0 | | 20.0 | | | |
| | 96 | 08 | 56.0 | | | | | | | | |
| | 98 | 55 | | 164.5 | | | 3.45 | | | | |
| | 105 | 23 | 68.5 | | | | | 20.4 | 3.6 | | |
| | 107 | 00 | | | | 66.0 | 3.69 | 20.0 | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 107 | 05 | 61.6 | 170.0 | | | | 20.0 | | | |
| | 112 | 45 | 82.0 | 177.0 | 0.0477 | | | 20.0 | | 6.75 | |
| 7 | 119 | 33 | 70.1 | | 0.0676 | | | 20.0 | 5.2 | | |
| | 121 | 59 | 65.5 | | 0.1002 | 74.0 | | 20.0 | | | |
| | 123 | 00 | | 167.5 | | | 3.41 | 20.0 | | | |
| | 123 | 25 | 59.8 | | 0.0337 | 54.0 | | | | | |
| | 127 | 50 | 72.1 | | 0.0478 | | | | | | |
| | 128 | 43 | 78.3 | | | | | | | | |
| | 129 | 15 | | | 0.0917 | 62.0 | | | | | |
| | 129 | 50 | | | | | 3.63 | | | | |
| | 129 | 55 | | 172.5 | | | | 20.9 | | | |
| | 130 | 50 | 72.4 | | | | | | | | |
| | 131 | 40 | | 169.0 | | | 3.58 | 21.0 | | | |
| | 137 | 10 | 74.3 | 174.5 | 0.0665 | | | 21.0 | | 6.75 | |
| Steady State | | | 64.2 | 167.5 | 0.0909 | 65.2 | 3.60 | 19.8 | 4.8 | 6.76 | |
| | 00 | 00 | 71.0 | | | | | | | | Dehydrogenase activities measured with 1 cm light path. |
| | 3 | 10 | 56.7 | | | | | | | | |
| | 5 | 37 | | | 0.0600 | | | | | | |
| 8 | 6 | 02 | | 165.0 | | | 3.70 | | | | |
| | 6 | 16 | 40.4 | | | | | | | | |
| | 7 | 34 | | | | | | | | | |
| | 195 | 26 | 74.3 | | | | | | | | |
| | 214 | 54 | | 225.0 | | | 3.71 | 20.5 | | | |
| | 215 | 08 | 99.5 | | | | | | | 6.70 | |
| | 216 | 10 | | | 0.0692 | | | | | | |
| | 218 | 41 | | 244.0 | | | 3.71 | | 5.2 | | |
| | 218 | 59 | 80.0 | | | | | | | | |
| | 221 | 29 | | 212.0 | | | 3.71 | | | | |
| | 222 | 24 | 86.9 | | | | | | | | |
| | 222 | 39 | | | | | | | | | |
| | 224 | 12 | 80.8 | | 0.0910 | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | 225 | 48 |  | 222.0 | 0.0783 |  | 3.60 |  |  |  |  |
|  | 226 | 00 | 78.4 |  | 0.1024 |  |  |  |  |  |  |
|  | 240 | 09 |  | 212.0 | 0.1487 |  | 3.95 |  |  |  |  |
|  | 240 | 20 | 85.8 |  | 0.0980 |  | 3.95 |  |  |  |  |
|  | 241 | 44 |  |  | 0.1487 |  |  |  |  |  |  |
|  | 244 | 44 | 83.8 |  | 0.1057 | 90.0 |  |  |  | 6.66 |  |
|  | 246 | 49 | 87.9 |  | 0.1409 |  |  |  | 5.3 |  |  |
|  | 247 | 09 |  | 226.0 |  |  |  |  |  |  |  |
|  | 247 | 38 | 91.8 |  | 0.1308 |  |  |  |  |  |  |
|  | 248 | 16 | 94.0 |  | 0.1284 | 96.0 |  |  |  |  |  |
|  | 249 | 14 |  | 229.5 |  |  | 3.98 |  |  |  |  |
|  | 249 | 30 |  |  | 0.1226 | 80.0 |  |  |  |  |  |
|  | 250 | 09 |  | 223.0 |  |  | 3.95 |  | 5.4 |  |  |
|  | 250 | 19 | 103.0 |  |  |  |  |  |  |  |  |
| Steady State |  |  | 85.4 | 222.9 | 0.1196 | 88.7 | 3.84 | 20.5 | 5.2 | 6.68 |  |
| 9 | 00 | 00 |  |  |  |  |  |  |  |  | Dehydrogenase activities measured with 1 cm cell. |
|  | 00 | 03 | 42.6 |  | 0.0758 |  |  |  |  |  |  |
|  | 1 | 00 |  | 165.0 |  |  |  |  |  |  |  |
|  | 1 | 37 | 55.3 |  | 0.1253 |  |  |  |  |  |  |
|  | 1 | 55 |  |  |  |  |  |  | 6.4 |  |  |
|  | 2 | 58 | 67.8 |  | 0.1054 |  |  |  |  |  |  |
|  | 3 | 13 |  | 179.0 |  |  |  |  |  | 6.76 |  |
|  | 3 | 23 |  |  |  |  | 4.54 |  |  |  |  |
|  | 6 | 20 | 86.0 |  | 0.0458 |  |  |  |  |  |  |
|  | 6 | 40 | 81.5 |  |  |  |  |  |  |  |  |
|  | 9 | 40 | 70.5 |  |  |  |  | 21.5 |  |  |  |
|  | 9 | 50 |  | 180.0 |  |  | 4.41 | 21.2 |  |  |  |
|  | 10 | 45 |  |  |  |  | 4.75 | 19.9 |  |  |  |
|  | 23 | 17 |  |  |  |  | 4.52 | 20.5 |  |  |  |
|  | 64 | 30 |  |  |  |  |  | 21.5 |  |  |  |
|  | 88 | 35 | 71.8 | 174.0 | 0.0555 |  |  | 20.0 |  |  |  |
|  | 118 | 12 | 69.4 |  |  |  |  | 22.0 |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 118 | 35 | | | | | | 22.0 | 5.2 | | |
| | 119 | 37 | 64.1 | | | | | 21.8 | | | |
| | 121 | 05 | 72.7 | | | | | 21.3 | | | |
| | 124 | 07 | 90.0 | | | | | 20.5 | | | |
| | 128 | 06 | 62.1 | | | | | | | | |
| | 139 | 15 | | 175.0 | 0.1345 | | | 20.0 | 4.8 | | |
| | 142 | 41 | 58.0 | | | | | | | | |
| 9 | 146 | 04 | 59.0 | | | | | | | | |
| | 149 | 00 | 79.0 | | | | | | | | |
| | 149 | 42 | 80.5 | | | | | | | | |
| | 152 | 50 | 76.0 | | 0.0750 | | | | | | |
| | 153 | 23 | 87.0 | | | | | | | | |
| | 154 | 00 | | 169.0 | | | 4.61 | | | 6.75 | |
| | 154 | 57 | 77.1 | | | | | 20.0 | | | |
| | 155 | 25 | | | 0.0583 | | | | | | |
| | 156 | 35 | | | | 70.0 | | | | | |
| | 156 | 57 | | 169.0 | | | 4.64 | 20.0 | | | |
| | 157 | 08 | 86.3 | | | | | | | | |
| | 157 | 30 | | | 0.0523 | | | | | | |
| | 158 | 11 | 86.9 | | | | | | | | |
| | 158 | 22 | | | | 52.0 | | | | | |
| | 159 | 10 | | 186.0 | | | 4.56 | 20.0 | | | |
| | 159 | 25 | 88.0 | | | | | | | | |
| | 164 | 30 | | 173.5 | | | 4.54 | | | | |
| | 164 | 45 | 84.0 | | | | | | | | |
| | 165 | 10 | 88.4 | | | | | | | | |
| | 165 | 19 | | | 0.0846 | | | | | | |
| | 166 | 48 | 78.2 | | 0.1392 | 80.0 | | 19.1 | | | |
| | 167 | 37 | | | | | 4.35 | 19.0 | | | |
| | 169 | 17 | 85.0 | | | 50.0 | | | | | |
| | 170 | 06 | 89.0 | | | | | | | | |
| | 170 | 17 | | 181.0 | 0.0873 | 52.0 | 4.58 | 19.5 | | | |
| 9 | 201 | 00 | | | | | | | | | |
| | 219 | 10 | 80.0 | | 0.0600 | | | | | 6.80 | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 220 | 43 | 79.0 |  |  |  |  |  |  |  |  |
|  | 221 | 17 |  |  |  |  | 4.65 |  |  |  |  |
|  | 221 | 55 |  | 179.2 |  |  |  | 20.2 |  |  |  |
|  | 222 | 10 | 85.5 |  |  | 74.0 |  |  |  |  |  |
|  | 223 | 02 |  | 179.2 |  |  | 4.61 |  |  |  |  |
|  | 223 | 58 | 94.8 |  | 0.0598 | 56.0 |  |  |  |  |  |
| Steady State |  |  | 87.6 | 176.8 | 0.0828 | 62.0 | 4.58 | 20.5 | 5.7 | 6.77 |  |
|  | 00 | 00 |  |  |  |  |  |  |  |  | Dehydrogenase activities measured with 1 cm light path. |
|  | 00 | 54 | 103.0 |  | 0.0223 |  |  |  |  |  |  |
|  | 1 | 52 |  | 186.0 |  |  | 4.98 |  |  |  |  |
|  | 2 | 09 | 101.0 |  |  |  |  |  |  | 6.8 |  |
|  | 4 | 00 | 102.0 |  |  |  |  | 20.8 |  |  |  |
|  | 4 | 30 |  | 185.5 | 0.0367 |  | 5.19 |  |  |  |  |
|  | 15 | 50 |  |  |  |  |  | 20.0 | 7.8 |  |  |
|  | 16 | 16 |  |  | 0.0526 |  |  |  |  |  |  |
|  | 16 | 33 | 113.4 |  |  |  |  |  |  |  |  |
|  | 16 | 48 | 109.0 |  | 0.0862 |  |  |  |  |  |  |
| 10 | 43 | 37 | 131.5 |  | 0.0888 |  |  |  |  |  |  |
|  | 43 | 50 | 128.0 |  |  |  |  |  |  |  |  |
|  | 46 | 48 |  |  | 0.0448 |  |  |  |  |  |  |
|  | 46 | 58 | 136.3 |  | 0.0372 |  |  |  |  |  |  |
|  | 48 | 03 | 129.7 |  | 0.0940 |  |  |  |  |  |  |
|  | 48 | 47 |  | 183.0 |  |  | 4.92 |  |  | 6.8 |  |
|  | 49 | 50 |  | 188.0 | 0.1002 |  | 4.89 | 20.0 |  |  |  |
|  | 50 | 04 | 133.5 |  |  |  |  |  |  |  |  |
| Steady State |  |  | 131.8 | 185.6 | 0.0691 | – | 5.00 | 20.2 | 7.8 | 6.8 |  |

Part B. Details of Steady State Runs with Galactose as Substrate

| Run No. | Time (hrs) | Time (min) | Glucose Concentrations Reactor (mg/ℓ) | Glucose Concentrations Influent (mg/ℓ) | Dehydrogenase Activity 1 cm cell (Absorbance) | Dehydrogenase Activity 10 cm cell (Absorbance) | Dry Solids Concentration (mg/ℓ) | Flow Rate (ℓ/hr) | Temp (°C) | DO (mg/ℓ) | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|  | 00 | 00 | 0.0 |  | 0.2418 |  |  |  |  |  |  |
|  | 28 | 33 | 1.0 |  | 0.1871 |  |  |  |  |  |  |
|  | 36 | 36 | 2.6 |  | 0.2396 |  |  |  |  |  |  |
|  | 40 | 21 |  | 130.2 |  |  |  | 0.922 | 20.5 |  |  |
|  | 44 | 31 |  | 127.5 |  |  | 84.0 | 0.968 |  | 1.6 | 6.80 |
|  | 46 | 31 |  |  | 0.0711 |  |  |  | 21.0 |  |  |
|  | 47 | 06 | 1.3 |  | 0.2262 |  |  |  | 23.0 |  | 6.80 |
|  | 50 | 16 |  |  | 0.0969 |  |  |  |  |  |  |
|  | 50 | 38 |  | 146.8 | 0.1024 |  |  | 0.910 |  |  |  |
|  | 51 | 26 | 4.8 |  | 0.1765 |  |  |  | 21.0 |  |  |
|  | 52 | 31 | 5.0 |  | 0.1746 |  | 90.0 |  | 20.9 |  |  |
|  | 54 | 17 | 3.5 | 136.3 |  |  |  |  |  |  |  |
| 1 | 55 | 21 |  |  | 0.2518 |  | 96.0 | 0.915 |  |  |  |
|  | 61 | 36 |  | 133.5 |  |  |  | 0.925 |  | 2.0 |  |
|  | 63 | 43 |  | 142.5 | 0.2306 |  |  |  | 23.0 |  | 6.80 |
|  | 64 | 23 | 4.1 |  |  |  |  |  |  |  |  |
|  | 65 | 05 |  |  |  |  |  | 0.970 |  |  |  |
|  |  |  |  | 141.5 | 0.1979 |  |  |  |  |  |  |
|  | 65 | 55 |  | 132.5 |  |  |  |  |  |  |  |
|  | 70 | 51 |  |  | 0.1871 |  |  |  |  |  |  |
| Steady State |  |  | 4.3 | 136.4 | 0.1541 |  | 90.0 | 0.935 | 21.6 | 2.0 | 6.80 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 00 | 00 | | 124.5 | | | | | 19.1 | | |
| | 1[illegible] | 03 | 1.8 | 133.0 | 0.0545 | 0.5702 | 60.0 | | | | |
| | 16 | 26 | | | | | | 0.939 | 20.0 | | |
| | 17 | 19 | 3.3 | 133.0 | | | | | | | |
| 2 | 20 | 38 | 1.7 | | 0.0610 | 0.5850 | 70.0 | | | | |
| | 20 | 50 | | 129.9 | | | | 0.938 | 20.0 | | |
| | 21 | 12 | 7.3 | | | | | | | | |
| | 22 | 00 | 3.5 | 135.0 | | | | | | 2.0 | 6.82 |
| | 23 | 25 | | | 0.0565 | 0.6472 | 68.0 | 0.974 | | | |
| | 23 | 27 | 6.2 | 135.8 | | | | | 20.0 | | |
| Steady State | | | 4.0 | 131.8 | 0.0573 | 0.6010 | 66.0 | 0.950 | 19.8 | 2.0 | 6.82 |
| | 00 | 00 | | | 0.1791 | | | | | | |
| | 2 | 12 | 2.4 | 192.2 | | | | | | | |
| | 3 | 02 | | | 0.1938 | | 102.0 | 1.515 | | | |
| | 3 | 35 | 1.3 | | 0.1349 | | | | 20.1 | | 6.6 |
| | 4 | 04 | | 171.5 | | | | 1.525 | | | |
| | 6 | 21 | | | 0.1487 | | | 1.510 | | | |
| | 17 | 40 | 0.8 | | 0.1844 | | 108.0 | | | | |
| 3 | 20 | 25 | | 181.0 | 0.1612 | | | 1.515 | 19.0 | | 6.80 |
| | 24 | 42 | 2.7 | | 0.1938 | | | | | | |
| | 25 | 05 | | 182.4 | | | | 1.415 | | | |
| | 26 | 27 | 3.0 | | | | 96.0 | | | | |
| | 26 | 49 | | 179.0 | 0.1805 | | | | | | |
| | 26 | 50 | 1.1 | | | | | 1.505 | 20.9 | | |
| Steady State | | | 1.9 | 131.2 | 0.1721 | — | 102.0 | 1.50 | 20.0 | 4.8 | 6.70 |
| | 00 | 00 | 5.83 | | | | | 1.781 | | | |
| | 17 | 51 | | 185.4 | | | | 1.740 | 19.7 | | |
| | 18 | 45 | 73.6 | | | 0.3468 | | | | | |
| 4 | 19 | 46 | | 177.2 | | | | 1.726 | 19.7 | | |
| | 21 | 30 | 53.0 | | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 23 | 36 | 67.7 | | | 0.2182 | | | | | |
| | 24 | 08 | | | | 0.2291 | | 1.787 | 19.9 | | 6.72 |
| | 24 | 09 | | 188.2 | | | | | | | |
| | 24 | 56 | 67.7 | | | | | | | | |
| 4 | 26 | 48 | | 186.8 | | | | 1.754 | 20.0 | | |
| | 27 | 38 | 71.3 | | | 0.2487 | 80.0 | | | | |
| | 27 | 55 | | 181.4 | | 0.2175 | | 1.760 | 20.0 | | |
| | 29 | 40 | 74.0 | | | 0.2924 | 61.0 | | | | |
| | 36 | 27 | | | | 0.4802 | 64.0 | | | | |
| | 37 | 23 | | 180.9 | | 0.2118 | | 1.775 | 19.8 | | |
| | 40 | 12 | 49.8 | | | | | | | | |
| | 40 | 23 | | 181.2 | | | | 1.784 | 20.2 | | |
| | 40 | 37 | 47.8 | | | | | | | | |
| | 41 | 21 | | | | | 74.0 | | | | |
| | 44 | 06 | 52.9 | | | | 79.0 | | | | |
| | 45 | 13 | 75.8 | | | | | | | | |
| | 46 | 00 | | | | | | 1.710 | 20.0 | | |
| | 46 | 14 | 46.2 | | | | | | | | |
| | 47 | 15 | 72.8 | | | 0.2020 | 76.0 | | | | |
| | 47 | 31 | | 189.8 | | | | 1.750 | 20.0 | | |
| | 47 | 50 | | | | | | | | 6.7 | 6.80 |
| | 48 | 27 | 71.2 | | | | | | | | |
| 4 | 48 | 58 | 74.5 | | | 0.2083 | | | | | |
| | 49 | 20 | | | | | 70.0 | | | | |
| | 49 | 40 | | 188.8 | | | | 1.750 | 20.0 | | |
| Steady State | | | 62.9 | 184.4 | — | 0.2655 | | 1.756 | 19.0 | 6.7 | 6.76 |
| | 00 | 00 | | 215.5 | 0.0264 | | | 1.92 | | | |
| | 0 | 50 | 83.0 | 172.0 | | | | | | | |
| | 3 | 13 | 85.0 | 152.0 | 0.1279 | | | | | | |
| | 4 | 58 | | 187.3 | | | | 1.94 | | | |
| | 5 | 30 | 87.7 | | 0.0943 | | | | 20.0 | | |
| | 6 | 37 | | 175.0 | | | | 1.96 | | | |
| 5 | 7 | 24 | 85.5 | | | | | | | | 6.80 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 8 | 08 | 90.3 |  | 0.0889 |  | 81.0 |  |  |  |  |
|  | 9 | 00 |  | 195.0 |  |  |  | 1.96 | 20.0 |  |  |
|  | 9 | 18 | 97.0 |  | 0.0494 |  |  |  |  |  |  |
|  | 10 | 13 |  |  | 0.0339 |  | 76.5 |  |  |  |  |
| 5 | 10 | 40 |  | 184.0 |  |  |  | 1.93 |  |  |  |
|  | 11 | 00 | 90.0 |  |  |  | 52.0 |  |  |  |  |
|  | 11 | 50 |  |  |  |  |  |  |  |  | 6.80 |
|  | 11 | 58 |  | 204.0 |  |  | 68.0 | 1.93 |  |  |  |
| Steady State |  |  | 87.7 | 185.6 | 0.0666 | — | 60.0 | 1.94 | 20.0 | 5.3 | 6.80 |
|  | 00 | 00 |  | 210.5 |  |  |  | 1.905 |  |  |  |
|  | 14 | 03 |  | 188.5 |  |  |  | 2.020 | 20.8 |  |  |
|  | 15 | 09 | 21.0 |  |  |  |  |  |  |  |  |
|  | 17 | 42 | 19.0 |  |  |  |  |  |  |  |  |
|  | 17 | 58 |  | 201.2 |  |  |  | 2.000 | 20.5 |  |  |
|  | 21 | 01 | 40.1 |  | 0.1701 |  | 92.0 |  |  |  | 6.72 |
|  | 23 | 21 |  |  |  |  |  | 2.000 | 20.5 |  |  |
|  | 23 | 43 |  | 195.5 |  |  |  | 2.010 |  |  |  |
|  | 23 | 48 |  |  | 0.1701 |  | 100.0 |  |  |  |  |
| 6 | 31 | 33 |  |  |  |  |  | 2.020 |  |  |  |
|  | 34 | 48 |  |  |  |  |  |  |  |  |  |
|  | 36 | 18 |  |  |  |  |  | 2.110 |  |  |  |
|  | 37 | 38 |  |  |  |  |  | 2.090 |  |  |  |
|  | 38 | 53 |  |  |  |  |  |  |  |  | 6.80 |
|  | 46 | 05 |  |  | 0.1805 |  | 107.5 |  |  |  |  |
|  | 46 | 55 |  |  |  |  |  |  |  |  |  |
|  | 47 | 19 | 26.8 | 191.5 |  |  |  | 2.070 |  |  |  |
|  | 47 | 58 |  |  |  |  |  | 2.000 | 20.5 |  |  |
|  | 52 | 03 |  |  |  |  |  | 2.000 |  |  |  |
|  | 52 | 58 |  |  | 0.1759 |  | 102.0 | 2.020 |  |  |  |
|  | 62 | 55 |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 26.8 | 197.4 | 0.1742 | — | 100.4 | 2.02 | 20.6 | 1.8 | 6.76 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 00 | 00 | | | | 0.2218 | | 2.29 | | | |
| | 0 | 40 | | | | | | | 20.7 | | |
| | 0 | 50 | | 170.0 | | | | | | | |
| | 4 | 59 | | 193.8 | | | | | 20.0 | | |
| | 5 | 07 | | 193.8 | | 0.2716 | | | | | |
| | 6 | 05 | | 170.0 | | 0.2218 | | 2.22 | 20.0 | | |
| 7 | 9 | 20 | | | | 0.5702 | 44.0 | | | | |
| | 9 | 45 | | 191.8 | | | | 2.29 | 20.0 | | |
| | 14 | 45 | 97.8 | | | 0.3188 | 46.0 | | | | |
| | 15 | 34 | | 187.5 | | | | 2.28 | 20.0 | | |
| | 15 | 45 | | | | 0.5072 | 48.0 | | | 6.2 | 6.75 |
| | 17 | 50 | 103.3 | | | | | | | | |
| | 18 | 05 | 89.0 | 172.5 | | 0.2899 | 28.0 | | | | |
| | 18 | 20 | | 192.7 | | | | | | | |
| | 18 | 32 | | | | | | 2.31 | 20.5 | | |
| | 19 | 01 | 116.5 | 184.5 | | | | | | | |
| | 20 | 05 | | | | 0.3575 | 44.0 | | | | |
| | 22 | 18 | | | | | | | | | |
| 7 | 23 | 22 | 103.0 | 177.0 | | | | | | | |
| | 23 | 40 | | 188.0 | | | | 2.26 | 20.5 | | |
| | 26 | 09 | 101.0 | 165.0 | | | 42.0 | | | | |
| | 27 | 00 | | | | | | 2.30 | 20.0 | | |
| | 27 | 06 | | 179.5 | | | 44.0 | | | | |
| | 27 | 16 | 114.6 | 168.0 | | | | | | | |
| | 27 | 36 | 115.4 | | | | | | | | |
| | 28 | 22 | | | | | 38.8 | | | | |
| | 28 | 56 | | | | | | | | | |
| | 29 | 40 | 110.0 | 181.0 | | 0.3298 | 38.8 | 2.31 | 20.0 | | |
| | 29 | 50 | | 169.0 | | | | | | | |
| | 30 | 38 | | 185.0 | | | | 2.32 | 20.5 | | |
| | 30 | 40 | 96.2 | 173.0 | | | | | | | |
| | 31 | 22 | 102.8 | | | | 27.7 | | | | |
| Steady State | | | 100.7 | 181.0 | — | 0.3502 | 40.1 | 2.29 | 20.2 | 6.2 | 6.75 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 00 | 00 | | | | | | 2.75 | 20.3 | | |
| | 0 | 19 | 94.5 | | | | | | | | |
| | 3 | 02 | | 191.8 | | | | | | | |
| | 3 | 17 | 108.3 | | | | | | | | |
| | 3 | 30 | | | | 0.1548 | 26.0 | | | | |
| | 4 | 02 | | | | | | 2.79 | 20.4 | | |
| 8 | 4 | 56 | 113.5 | | | 0.1581 | | | | | |
| | 8 | 39 | 130.8 | | | 0.0878 | | | | | |
| | 10 | 01 | | 192.8 | | | | 2.72 | 20.0 | | |
| | 10 | 42 | | | | 0.1074 | 27.8 | | | | |
| | 11 | 48 | 112.6 | | | | | | | | |
| | 13 | 20 | | | | 0.0949 | | | | | |
| | 14 | 17 | | 187.2 | | | | 2.72 | 20.0 | | |
| | 14 | 47 | 119.0 | | | | | | | | |
| | 16 | 35 | 121.0 | | | 0.0840 | | | | | |
| | 17 | 50 | 113.7 | | | 0.0732 | 26.0 | | | | |
| | 18 | 30 | | 183.3 | | | | 2.77 | 20.0 | | |
| | 20 | 35 | 125.4 | | | 0.0946 | | | | | |
| 8 | 21 | 55 | 117.4 | | | | 26.4 | | | | |
| | 22 | 42 | | 184.6 | | | | 2.75 | 20.0 | | |
| | 22 | 50 | | | | | | | | 6.7 | 6.8 |
| | 23 | 39 | 114.6 | | | | | | | | |
| | 23 | 53 | | | | | 29.0 | | | | |
| | 24 | 40 | | | | | | 2.71 | 20.0 | | |
| | 24 | 45 | | 184.0 | | | 25.0 | | | | |
| Steady State | | | 116.2 | 187.3 | — | 0.1068 | 26.7 | 2.74 | 20.1 | 6.7 | 6.8 |
| | 0 | 0 | 7.7 | | 0.1002 | | | 2.76 | | | |
| | 1 | 26 | | 177.8 | | | | | | | |
| 9 | 2 | 42 | | | 0.0757 | | | | | | |
| | 5 | 52 | | | 0.0862 | | | | | | |
| | 6 | 26 | | 165.0 | | | | 2.92 | | | |
| | 7 | 55 | 9.0 | | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 8 | 06 |  | 172.6 |  |  |  | 2.87 |  |  |  |
| 9 | 19 | 12 |  |  |  |  |  | 2.78 | 20.5 |  | 6.73 |
|  | 19 | 58 |  | 170.8 | 0.0800 |  |  |  |  |  |  |
|  | 20 | 36 |  |  |  |  |  |  |  |  |  |
|  | 23 | 05 |  |  | 0.1669 |  |  |  |  |  |  |
|  | 24 | 58 |  | 154.6 |  |  |  | 2.84 |  |  |  |
|  | 26 | 41 |  |  |  |  |  |  |  |  |  |
|  | 30 | 11 | 13.5 |  |  |  |  |  |  |  |  |
|  | 30 | 44 |  |  |  |  |  |  |  |  |  |
|  | 32 | 37 | 17.0 |  |  |  |  |  |  |  |  |
|  | 33 | 04 |  | 167.0 |  |  |  | 2.84 |  |  |  |
|  | 34 | 39 | 11.7 |  |  |  |  |  |  |  |  |
|  | 35 | 53 |  |  | 0.0902 |  |  |  |  |  |  |
|  | 36 | 36 |  |  |  |  |  | 2.78 |  |  |  |
|  | 38 | 00 | 18.3 |  |  |  |  |  |  |  |  |
|  | 38 | 41 |  |  |  |  |  | 2.74 | 20.5 |  |  |
|  | 39 | 01 |  |  | 0.0849 |  |  |  |  |  |  |
|  | 40 | 55 | 27.8 |  |  |  |  |  |  |  |  |
|  | 41 | 08 |  |  |  |  |  |  |  |  |  |
|  | 43 | 31 | 23.8 |  |  |  | 64.0 |  |  |  | 6.77 |
|  | 57 | 49 | 11.0 |  | 0.0810 |  |  |  |  |  |  |
|  | 58 | 26 |  | 164.0 |  |  |  | 2.78 | 20.5 |  |  |
|  | 59 | 02 |  | 149.7 |  |  |  |  |  |  |  |
|  | 59 | 23 | 18.4 |  | 0.0862 |  | 62.0 |  |  |  |  |
|  | 60 | 11 | 17.2 |  |  |  |  |  |  |  |  |
|  | 61 | 43 |  |  |  |  |  | 2.83 | 20.0 |  |  |
|  | 61 | 52 | 20.5 |  |  |  |  |  |  |  |  |
|  | 61 | 53 |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 24.0 | 165.2 | 0.0946 | — | 63.0 | 2.81 | 20.4 | 4.8 | 6.75 |
|  | 0 | 0 |  |  |  |  | 48.0 |  | 18.5 |  |  |
|  | 0 | 57 |  | 182.0 |  |  |  |  |  |  |  |
| 10 | 2 | 12 | 145.0 |  | 0.0177 |  |  |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 57 | | 203.0 | | | 40.0 | 3.05 | | | |
| | 4 | 01 | 143.0 | | 0.0200 | 0.1871 | | | | | |
| | 4 | 57 | | | | 0.2090 | | 2.95 | | | |
| | 4 | 58 | 181.0 | | 0.0223 | 0.0969 | | | 20.5 | | |
| 10 | 7 | 34 | | | | 0.0841 | | | | | |
| | 8 | 22 | | 212.0 | | 0.1337 | 32.0 | 2.98 | | | |
| | 9 | 28 | 163.0 | | 0.0246 | 0.1319 | | | | | |
| | 10 | 27 | 189.0 | | | 0.1307 | | | | | |
| | 11 | 14 | | 185.0 | | | | 3.00 | 19.5 | | 7.1 |
| | 11 | 25 | 158.0 | | | | 26.7 | | | | |
| | 12 | 21 | | 187.0 | | 0.1018 | | 2.96 | | | |
| Steady State | | | 172.8 | 193.8 | 0.0212 | 0.1344 | 29.4 | 2.99 | 19.5 | 6.0 | 7.1 |
| | 0 | 0 | 86.5 | | | | | | | | |
| | 2 | 38 | | 213.0 | | | | 3.49 | | | |
| | 6 | 47 | 131.5 | | 0.0931 | | | | | | |
| | 9 | 07 | 120.5 | | 0.1308 | | | | | | |
| | 9 | 26 | | | | | | 3.32 | | | 6.94 |
| | 9 | 30 | 112.2 | | 0.1308 | | | | | | |
| | 10 | 08 | | | | | | | | | |
| | 11 | 32 | 87.5 | | | | | | | | |
| | 11 | 41 | | 234.0 | | | | 3.49 | | | |
| | 12 | 36 | 66.1 | | | | | | | | |
| | 12 | 55 | | 273.5 | | | | 3.54 | | | |
| | 14 | 22 | | | 0.1107 | | | | | | |
| | 15 | 05 | | 269.0 | | | | 3.49 | 22.0 | | 6.92 |
| | 16 | 26 | | | 0.0969 | | | | | | |
| | 18 | 15 | 117.0 | 244.0 | 0.0921 | | | 3.58 | 22.0 | | |
| | 23 | 18 | 123.4 | | | | | | | | |
| | 24 | 26 | 120.0 | | | | | | | | |
| | 25 | 48 | | | 0.0921 | | | | | | |
| 11 | 26 | 11 | | 258.0 | | | | 3.70 | | | |
| | 26 | 58 | | | | | | 3.56 | 22.0 | | 6.82 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 27 | 08 | 110.0 |  | 0.0675 |  | 51.2 |  |  |  |  |
|  | 27 | 33 |  |  |  |  |  |  | 21.0 |  |  |
|  | 28 | 04 | 120.0 |  |  |  |  |  | 20.3 |  |  |
|  | 32 | 08 |  | 267.0 | 0.0850 |  |  | 3.59 |  |  |  |
|  | 32 | 37 | 119.0 |  |  |  |  |  |  |  |  |
|  | 33 | 10 |  |  |  |  | 54.0 |  |  |  |  |
|  | 33 | 40 | 133.0 |  |  |  |  |  |  |  |  |
|  | 33 | 53 |  | 249.0 |  |  |  | 3.50 | 20.0 |  |  |
|  | 34 | 20 | 128.0 |  |  |  | 51.2 |  |  |  |  |
|  | 35 | 22 | 134.7 |  |  |  |  |  |  |  |  |
|  | 35 | 28 |  | 252.0 |  |  |  | 3.50 | 20.0 |  | 6.92 |
|  | 52 | 18 | 134.0 |  |  |  | 52.0 |  |  |  |  |
| Steady State |  |  | 133.9 | 255.8 | 0.0958 | — | 52.1 | 3.52 | 21.0 | 4.1 | 6.90 |

Part C. Details of Steady State Runs with Glucose and Galactose Mixtures as Substrates

| Run No. | Time |  | Glucose Concentrations |  | Galactose Concentrations |  | Dehydrogenase Activity |  | Dry Solids Concentration | Flow Rate | Temp | DO | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  | Influent | Reactor | Influent | Reactor | 1 cm cell | 10 cm cell |  |  |  |  |  |
|  | (hrs)- | (min) | (mg/ℓ) | (mg/ℓ) | (mg/ℓ) | (mg/ℓ) | (O.d.) | (o.d.) | (mg/ℓ) | (ℓ/hr) | (°C) | (mg/ℓ) |  |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|  | 0 | 0 |  | 0.7 |  | 3.9 | 0.1531 |  |  |  |  |  |  |
|  | 0 | 28 |  |  |  | 6.9 |  |  |  |  | 20.6 |  |  |
|  | 1 | 19 |  |  |  | 3.2 |  |  |  |  |  |  |  |
|  | 1 | 26 | 107.3 |  |  |  |  |  |  | 2.095 | 20.5 |  |  |
|  | 2 | 28 |  |  |  |  |  |  |  | 2.005 |  |  | 6.8 |
|  | 3 | 03 |  |  |  | 7.4 |  |  |  |  |  |  |  |
|  | 4 | 48 | 118.4 |  |  |  |  |  |  | 2.004 | 19.4 |  |  |
|  | 5 | 28 |  |  |  |  |  |  |  | 2.095 |  |  |  |
|  | 7 | 28 | 116.9 |  |  |  |  |  |  | 1.980 | 19.5 |  |  |
|  | 8 | 35 |  | 2.5 |  |  | 0.1491 |  |  |  |  |  |  |
|  | 10 | 10 |  | 1.1 |  |  | 0.1769 |  |  |  |  |  |  |
| 1 | 10 | 56 | 125.3 |  |  |  |  |  |  | 1.930 | 19.1 |  |  |
|  | 15 | 38 | 109.3 |  |  |  |  |  |  | 2.000 |  |  |  |
|  | 18 | 08 |  | 0.5 |  |  | 0.1991 |  |  |  |  |  |  |
|  | 22 | 58 |  | 0.5 |  |  |  |  |  |  |  |  |  |
|  | 23 | 48 |  |  |  |  |  |  |  | 1.980 |  |  |  |
|  | 27 | 45 | 106.7 |  |  |  |  |  |  | 1.924 | 20.0 |  |  |
|  | 28 | 12 |  | 0.2 |  | 1.3 | 0.1251 |  |  |  |  |  |  |
|  | 29 | 20 |  |  |  |  |  |  |  | 2.015 |  |  |  |
|  | 29 | 24 | 113.2 |  |  |  |  |  |  | 1.990 |  | 2.0 |  |
|  | 30 | 24 |  |  |  |  |  |  |  |  |  |  |  |
|  | 31 | 01 |  | 0.2 |  |  | 0.1094 |  |  |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 33 | 33 |  |  |  |  |  |  |  |  |  |  |  |
|  | 33 | 43 |  |  |  |  |  |  |  |  | 19.5 |  |  |
|  | 36 | 30 |  | 6.6 |  | 0.8 | 0.1760 |  |  |  |  |  |  |
|  | 37 | 30 |  |  |  |  |  |  |  |  |  |  |  |
|  | 39 | 27 |  | 6.3 |  | 0.7 | 0.1803 |  | 164.0 |  |  |  |  |
|  | 40 | 18 |  |  |  |  |  |  |  | 2.130 | 19.3 |  |  |
|  | 40 | 23 |  |  | 114.5 |  |  |  |  |  |  |  |  |
|  | 40 | 40 |  | 7.0 |  | 0.8 | 0.1868 |  | 136.0 |  |  |  |  |
|  | 41 | 49 |  | 9.4 |  |  | 0.1612 |  | 122 |  |  |  |  |
|  | 42 | 40 |  |  |  |  |  |  |  |  |  |  |  |
|  | 43 | 43 |  |  | 125.3 |  | 0.1593 |  | 114.0 | 2.160 | 20.2 |  |  |
| 1 | 54 | 48 |  |  |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 113.9 | 3.2 | 119.9 | 3.1 | 0.1615 | — | 114.0 | 2.02 | 19.3 | 2.0 | 6.8 |
|  | 0 | 0 |  |  |  |  |  |  |  | 2.73 |  |  |  |
|  | 2 | 23 |  |  | 165.0 |  |  |  |  | 2.55 | 20.3 |  |  |
|  | 7 | 27 |  |  | 147.0 |  |  |  |  | 2.69 |  |  |  |
|  | 8 | 44 |  |  |  |  | 0.1878 |  |  |  |  |  |  |
|  | 18 | 03 |  | 2.1 |  |  | 0.1521 |  |  |  |  |  |  |
|  | 18 | 53 | 118.9 |  | 145.6 |  |  |  |  | 2.68 | 19.1 |  | 6.76 |
|  | 19 | 20 |  | 1.7 |  |  | 0.1282 |  |  |  |  |  |  |
|  | 20 | 23 |  | 1.4 |  |  |  |  |  |  |  |  |  |
|  | 21 | 43 | 122.8 |  | 147.5 |  |  |  |  | 2.58 | 19.8 |  |  |
|  | 41 | 41 |  |  |  |  |  |  |  |  | 19.5 |  |  |
|  | 112 | 18 |  | 5.8 |  |  |  |  |  |  |  |  |  |
|  | 116 | 21 |  |  | 164.0 |  |  |  |  | 2.56 | 18.9 |  |  |
|  | 119 | 45 |  |  |  |  |  |  |  |  | 20.0 |  |  |
|  | 136 | 19 |  | 0.5 |  | 15.1 |  |  |  |  |  |  |  |
|  | 136 | 18 |  |  |  |  |  |  |  |  |  |  |  |
| 2 | 138 | 28 |  |  |  |  | 0.1647 |  |  |  |  |  |  |
|  | 139 | 13 |  |  |  |  |  |  |  | 2.62 | 20.4 |  |  |
|  | 140 | 30 |  |  |  |  | 0.1282 |  |  |  |  |  |  |
|  | 141 | 13 | 120.5 |  | 149.0 |  |  |  |  | 2.60 | 20.0 |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 141 | 38 | | 1.4 | | 25.8 | | | | | | | |
| | 143 | 13 | | | 157.3 | | | | | 2.58 | 20.0 | | |
| | 144 | 45 | | 1.5 | | | | | | | | | |
| | 144 | 55 | | | | 40.1 | 0.1130 | | | | | | |
| | 148 | 21 | | 1.1 | | 36.2 | 0.1293 | | | | | | |
| | 148 | 43 | | | 156.7 | | | | | 2.48 | | | |
| | 149 | 03 | | | | | | | | 2.66 | 20.0 | | |
| | 149 | 29 | | 0.9 | | 37.4 | | | | | | | 6.80 |
| | 149 | 42 | | | | | 0.1676 | | 110 | | | | |
| | 153 | 51 | | 2.5 | | 44.8 | | | | | | | |
| | 154 | 01 | | | | | 0.1090 | | 92.0 | | | | |
| | 154 | 25 | | 2.0 | | 27.6 | | | | | | | |
| | 154 | 43 | | | | | 0.1615 | | 128.0 | | | | |
| 2 | 154 | 50 | | | 148.2 | | | | | 2.68 | 20.1 | | |
| | 159 | 25 | | | | | | | | | | 3.6 | |
| Steady State | | | 120.7 | 1.7 | 153.4 | 37.2 | 0.1441 | — | 110.0 | 2.62 | 19.8 | 3.6 | 6.78 |
| | 0 | 0 | | | 186.9 | | | | | 2.84 | 19.6 | | |
| | 0 | 52 | | | | | | | | | | | |
| | 1 | 14 | | | | | | | | 2.84 | 20.0 | | |
| | 5 | 28 | | | | | | | | 2.86 | 19.6 | | |
| | 7 | 26 | | 8.0 | | | | | | | | | |
| | 8 | 23 | | 6.6 | | | | | | | | | |
| 3 | 9 | 23 | | 9.9 | | | | 0.29[illegible]4 | | | | | |
| | 11 | 40 | | | 181.5 | | | | | | | | |
| | 13 | 00 | | 6.0 | 194.4 | 72.0 | | | | | | | |
| | 13 | 33 | | | | | 0.0704 | | | | | | 6.8 |
| | 14 | 48 | | 4.6 | | 64.4 | 0.0933 | | | | | | |
| | 15 | 39 | 135.2 | | 186.1 | | | | | 2.92 | | | |
| | 17 | 35 | | | | 59.9 | 0.0753 | | | | | | |
| | 20 | 01 | | | | | | | | 2.96 | 20.4 | | |
| | 20 | 03 | 124.7 | | 197.5 | | | | | | | | |
| | 23 | 07 | | 4.3 | | 61.0 | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 23 | 16 | | | | | | 0.3059 | | | | | |
| | 23 | 53 | 126.3 | | 180.4 | | | | | 2.86 | 20.1 | | |
| | 26 | 08 | | 6.9 | | 70.9 | | | | | | | |
| | 26 | 25 | | | | | | | | | | | |
| | 36 | 48 | | 4.8 | | | | | | | | | |
| | 37 | 06 | | | | | 0.0875 | | | | | | |
| | 37 | 45 | | | | | | 0.2774 | | | | | |
| | 45 | 48 | 130.0 | | 196.4 | | | | | 2.92 | 20.1 | | |
| | 46 | 15 | | | | 61.5 | | | | | | | |
| | 46 | 53 | | | | | | | | | | | |
| | 48 | 23 | | | | 52.5 | | | | 2.92 | | | 6.8 |
| | 48 | 33 | | | 178.0 | | | | | | | | |
| | 49 | 00 | | | | | | | | | | | |
| | 50 | 14 | | 4.4 | | 61.1 | | | | | | | |
| | 50 | 28 | | | | | | | | | | | |
| | 51 | 19 | | | | 64.1 | 0.1145 | | | | | | |
| | 52 | 28 | | | | | | | | 2.84 | 19.7 | | |
| | 53 | 20 | 131.0 | | 186.3 | 72.3 | | | | | | | |
| | 54 | 01 | | | | | | | | 2.85 | 19.8 | | |
| | 55 | 19 | | | | 65.5 | | | | | | | |
| | 57 | 21 | | | | 68.1 | | 0.4933 | | | | | |
| | 60 | 18 | | | | | | | | | 20.8 | | |
| | 61 | 02 | | | | 66.4 | | 0.3947 | | | | | |
| | 62 | 28 | 129.0 | | 175.0 | | | | | 2.93 | 20.5 | | |
| | 63 | 34 | | | | 69.1 | 0.0905 | | | | | | |
| | 64 | 20 | 131.3 | | 176.4 | | | | | 2.91 | 20.0 | | |
| 3 | 64 | 48 | | 5.2 | | 74.2 | 0.0763 | | 122.0 | | | | |
| | 66 | 33 | 124.5 | | 184.0 | | | | | 2.95 | 20.0 | | |
| | 66 | 45 | | | | | | | 117.0 | | | | |
| | 67 | 41 | | 7.4 | | 79.5 | | 0.4118 | 97.5 | | | | |
| | 68 | 06 | | | | | 0.0789 | 0.6295 | 138.0 | | | | |
| | 68 | 24 | | | | | | | | | 20.0 | | |
| | 69 | 24 | 130.0 | | 184.3 | | | | | | | | |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 69 | 33 |  | 4.7 |  | 68.6 |  |  |  |  |  |  |  |
| 3 | 69 | 38 |  |  |  |  |  |  |  |  |  | 4.0 |  |
| Steady State |  |  | 129.1 | 5.5 | 185.2 | 67.9 | 0.0858 | 0.4004 | 118.6 | 2.85 | 20.1 | 4.0 | 6.8 |
|  | 0 | 0 |  | 11.8 |  | 136.3 |  |  |  |  |  |  |  |
|  | 1 | 0 |  |  |  |  |  |  |  |  |  |  |  |
|  | 18 | 45 |  | 50.8 |  |  | 0.0288 |  |  |  |  |  |  |
|  | 21 | 10 |  | 46.0 |  | 136.0 | 0.0339 |  |  |  |  |  |  |
|  | 21 | 29 | 139.1 |  | 172.5 |  |  |  |  | 3.35 | 20.0 |  |  |
|  | 23 | 53 |  | 50.0 |  |  | 0.0377 | 0.2031 |  |  |  |  |  |
|  | 26 | 58 | 140.4 |  | 172.6 |  |  |  |  | 3.25 | 20.0 |  |  |
|  | 27 | 26 |  | 49.9 |  | 124.2 | 0.0438 | 0.2845 |  |  |  |  |  |
|  | 28 | 47 |  | 35.1 |  | 127.0 |  |  |  |  |  |  | 6.74 |
| 4 | 29 | 10 | 149.2 |  | 166.9 |  |  |  |  | 3.37 | 20.0 |  |  |
|  | 31 | 08 |  | 42.0 |  | 128.3 | 0.0311 | 0.2066 |  |  |  |  |  |
|  | 32 | 07 | 144.4 |  | 162.4 |  |  |  |  | 3.32 | 20.5 |  |  |
|  | 33 | 47 |  | 33.4 |  |  | 0.0427 | 0.2702 | 82.0 |  |  |  |  |
|  | 34 | 09 | 159.0 |  | 158.0 |  |  |  |  | 3.29 | 20.2 |  |  |
|  | 35 | 46 |  | 41.1 |  | 128.0 | 0.0334 | 0.2090 | 92.0 |  |  |  |  |
|  | 36 | 35 | 150.8 |  | 167.5 |  |  |  |  | 3.31 | 20.0 |  |  |
|  | 36 | 48 |  |  |  |  |  |  |  |  |  | 5.0 |  |
|  | 37 | 09 |  | 41.7 |  | 124.0 |  |  |  |  |  |  |  |
|  | 37 | 35 | 153.1 |  | 162.5 |  |  |  |  | 3.31 |  |  |  |
|  | 38 | 46 |  |  |  |  |  |  | 94.0 |  |  |  |  |
|  | 53 | 38 |  | 43.6 |  | 120.0 |  |  |  |  |  |  |  |
|  | 54 | 05 |  | 44.4 |  | 127.4 | 0.0264 |  |  |  |  |  |  |
| 4 | 59 | 38 |  | 38.0 |  |  | 0.0304 | 0.2577 |  |  |  |  |  |
|  | 61 | 04 |  | 46.0 |  |  | 0.0121 | 0.1683 |  |  |  |  | 6.82 |
|  | 62 | 30 |  |  |  |  |  |  |  |  |  | 5.7 |  |
|  | 62 | 31 | 150.0 |  | 160.0 |  |  |  |  | 3.28 | 20.0 |  |  |
|  | 62 | 38 |  |  |  |  |  |  | 86.0 | 3.45 |  |  |  |
|  | 62 | 55 |  |  |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 151.1 | 44.9 | 165.0 | 127.9 | 0.03312 | 0.2285 | 88.5 | 3.32 | 20.1 | 5.35 | 6.78 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 0 | 0 |  |  |  |  |  |  |  | 3.47 |  |  |  |
|  | 0 | 28 |  | 48.0 |  | 150.0 |  | 0.2285 |  |  |  |  |  |
|  | 0 | 48 |  |  |  |  |  |  |  |  |  |  |  |
|  | 0 | 53 |  |  |  |  |  |  |  |  |  |  |  |
|  | 1 | 18 | 147.0 |  | 191.5 |  |  |  |  | 3.61 |  |  |  |
|  | 1 | 28 |  | 56.5 |  | 150.0 |  |  |  |  |  |  |  |
|  | 1 | 46 |  | 73.4 |  | 142.5 |  | 0.2027 |  |  |  |  |  |
|  | 4 | 24 |  | 78.5 |  | 157.5 |  | 0.1622 |  |  |  |  | 6.8 |
|  | 4 | 45 |  |  |  |  |  |  |  |  |  | 6.6 |  |
|  | 4 | 50 | 160.5 |  | 192.9 |  |  |  |  | 3.46 | 19.2 |  |  |
|  | 5 | 10 |  |  |  |  |  |  |  | 3.57 |  |  |  |
|  | 5 | 34 |  | 77.5 |  | 152.5 |  |  |  |  |  |  |  |
| 5 | 7 | 32 |  | 82.7 |  | 153.0 |  |  |  |  |  |  |  |
|  | 7 | 55 | 155.4 |  | 181.9 |  |  |  |  | 3.38 |  |  |  |
|  | 8 | 52 | 161.0 |  | 189.8 |  |  |  |  | 3.54 |  |  |  |
|  | 9 | 12 |  | 83.1 |  | 146.5 |  | 0.1218 |  |  |  |  |  |
|  | 12 | 32 |  | 83.0 |  | 178.5 |  |  |  |  |  |  |  |
|  | 12 | 58 | 152.4 |  | 186.7 |  |  |  |  | 3.54 | 20.0 |  |  |
|  | 15 | 03 |  | 61.1 |  | 160.6 |  |  | 64.0 |  |  |  |  |
|  | 15 | 28 | 160.5 |  | 194.3 |  |  |  |  | 3.57 | 20.4 |  |  |
|  | 15 | 57 |  | 76.7 |  | 158.4 |  |  |  |  |  |  |  |
|  | 16 | 45 | 159.0 |  | 192.8 |  |  |  |  | 3.59 |  |  |  |
|  | 16 | 50 |  | 65.6 |  | 145.0 |  |  |  |  |  |  |  |
|  | 17 | 35 |  | 73.5 |  | 140.0 |  |  | 80.0 |  |  |  |  |
|  | 18 | 37 | 150.2 |  | 194.6 |  |  |  |  | 3.57 | 20.7 |  |  |
|  | 21 | 20 |  |  | 190.8 |  |  |  |  | 3.40 |  |  |  |
|  | 22 | 33 | 160.6 |  | 184.5 |  |  |  |  | 3.54 | 20.2 |  |  |
|  | 23 | 50 | 157.0 |  | 187.0 |  |  |  | 70.0 | 3.47 |  |  |  |
|  | 25 | 13 |  | 64.3 |  | 149.6 |  |  |  |  |  |  |  |
|  | 25 | 30 | 157.0 |  | 187.0 |  |  |  |  | 3.49 |  |  |  |
|  | 27 | 55 |  |  |  |  |  |  |  |  |  |  |  |
| 5 | 29 | 17 | 163.9 |  | 201.6 |  |  |  |  | 3.49 | 20.4 |  |  |
|  | 30 | 58 |  | 71.3 |  | 176.6 |  | 0.1721 |  |  |  |  |  |
|  | 31 | 37 |  |  |  |  |  |  | 60.0 |  |  |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 31 | 55 |  |  | 183.0 |  |  | 0.1576 |  | 3.50 |  |  |  |
|  | 32 | 13 |  |  |  |  |  |  | 74.0 |  |  |  |  |
|  | 32 | 16 |  | 72.8 |  | 172.0 |  |  |  |  |  |  |  |
| Steady State |  |  | 157.0 | 74.1 | 190.5 | 150.5 | — | 0.1741 | 69.6 | 3.51 | 20.2 | 6.45 | 6.8 |
|  | 0 | 0 |  |  |  |  |  |  |  |  |  |  |  |
|  | 0 | 12 |  |  |  |  |  |  |  | 3.89 |  |  |  |
|  | 0 | 55 |  | 79.5 |  | 177.5 |  | 0.3923 |  |  |  |  |  |
|  | 1 | 40 |  | 85.0 |  | 153.2 |  | 0.1721 |  |  |  |  |  |
|  | 1 | 59 | 163.5 |  | 195.4 |  |  |  |  | 3.86 | 20.0 |  |  |
|  | 2 | 27 |  | 84.2 |  | 167.5 |  | 0.1207 |  |  |  |  |  |
|  | 4 | 14 |  | 91.3 |  | 164.4 |  | 0.0757 |  |  |  | 6.40 | 6.76 |
|  | 14 | 25 |  |  |  | 169.4 |  | 0.0939 |  |  |  |  |  |
|  | 16 | 16 |  | 76.2 |  |  |  |  | 76.0 |  |  |  |  |
| 6 | 16 | 37 | 159.6 |  | 184.4 |  |  |  |  | 3.86 | 20.9 |  |  |
|  | 18 | 50 | 163.2 |  | 200.0 |  |  |  |  | 3.96 | 20.0 |  |  |
|  | 19 | 03 |  | 64.9 |  | 166.7 |  |  |  |  |  |  |  |
|  | 19 | 58 |  | 80.0 |  | 168.0 |  |  |  |  |  |  |  |
|  | 22 | 24 |  | 88.3 |  | 163.4 |  |  |  |  |  |  |  |
|  | 22 | 50 | 165.5 |  | 200.0 |  |  |  | 72.0 | 3.91 |  |  |  |
|  | 24 | 38 |  | 89.2 |  | 164.0 |  | 0.1008 |  |  |  |  |  |
|  | 26 | 14 |  | 93.0 |  | 179.0 |  | 0.0866 |  |  |  |  |  |
|  | 27 | 17 |  | 92.5 |  | 172.7 |  |  | 64.0 |  |  |  |  |
|  | 28 | 00 | 169.3 |  | 208.0 |  |  |  |  | 3.88 |  |  |  |
|  | 29 | 52 |  | 86.4 |  | 174.0 |  | 0.1024 | 72.0 |  |  |  |  |
|  | 30 | 38 | 159.5 |  | 203.5 |  |  |  | 68.0 | 3.91 | 21.0 |  |  |
| 6 | 32 | 35 |  | 83.4 |  | 165.0 |  |  | 80.0 |  |  |  |  |
|  | 33 | 09 |  | 72.5 |  | 163.0 |  |  |  |  |  |  | 6.80 |
| Steady State |  |  | 163.4 | 85.8 | 201.4 | 167.7 | — | 0.1320 | 72.0 | 3.90 | 20.5 | 6.40 | 6.78 |
|  | 0 | 0 |  | 104.9 |  | 172.4 |  |  |  |  |  |  | 6.84 |
| 7 | 0 | 46 |  | 102.9 |  | 164.2 |  |  |  |  |  |  |  |
|  | 1 | 25 | 170.0 |  | 189.0 |  |  |  |  | 4.38 | 20.7 |  |  |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 3 | 12 |  | 104.4 |  | 169.5 |  |  |  |  |  |  |  |
|  | 3 | 38 | 170.0 |  | 188.4 |  |  |  |  | 4.42 | 21.4 |  |  |
|  | 5 | 03 |  |  | 188.4 |  |  |  |  | 4.39 | 20.7 |  |  |
|  | 5 | 47 |  |  |  |  |  |  |  |  |  |  |  |
|  | 6 | 01 |  | 103.0 |  | 168.4 |  | 0.1404 |  |  |  |  |  |
|  | 7 | 30 |  | 106.1 |  |  |  |  |  |  |  |  |  |
|  | 7 | 40 |  |  |  | 184.0 |  |  |  |  |  |  |  |
|  | 7 | 57 | 167.2 |  | 190.0 |  |  |  |  | 4.39 |  |  |  |
|  | 8 | 52 |  |  |  |  |  |  | 52.0 |  |  |  |  |
|  | 10 | 27 |  | 100.0 |  | 177.2 |  |  |  |  |  |  |  |
|  | 10 | 44 |  |  |  |  |  | 0.1167 | 52.0 |  |  |  |  |
|  | 11 | 38 | 172.0 |  | 212.0 |  |  |  |  | 4.38 |  |  |  |
|  | 12 | 14 |  | 103.6 |  | 190.0 |  |  | 54.0 |  |  |  |  |
|  | 12 | 49 |  |  |  |  |  | 0.1854 | 52.0 |  |  |  |  |
|  | 13 | 15 |  | 112.0 |  | 195.0 |  |  |  |  |  |  |  |
|  | 13 | 58 |  |  |  |  |  | 0.2218 | 58.0 |  |  |  |  |
|  | 14 | 14 |  | 113.5 |  | 195.0 |  |  | 50.0 |  |  |  |  |
|  | 14 | 43 | 160.0 |  | 196.0 |  |  |  |  | 4.43 | 20.0 | 6.30 |  |
|  | 15 | 27 |  |  |  |  |  | 0.1568 | 46.0 |  |  |  |  |
|  | 15 | 54 |  | 115.2 |  | 188.4 |  |  |  |  |  |  |  |
|  | 16 | 27 | 172.0 |  | 198.0 |  |  |  |  | 4.40 |  |  | 6.80 |
|  | 17 | 59 |  |  |  |  |  | 0.1389 | 44.0 |  |  |  |  |
|  | 19 | 44 | 172.5 |  | 200.0 |  |  |  | 42.0 | 4.41 |  |  |  |
|  | 20 | 03 |  | 110.2 |  | 201.5 |  | 0.2145 | 44.0 |  |  |  |  |
|  | 22 | 21 | 160.0 |  | 200.0 |  |  |  |  | 4.42 | 21.0 |  |  |
|  | 22 | 29 |  |  |  |  |  |  |  |  |  |  |  |
| Steady State |  |  | 168.0 | 106.9 | 195.7 | 194.0 | -- | 0.1678 | 52.0 | 4.402 | 20.8 | 6.30 | 6.82 |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | 0 | 0 |  | 129.7 |  | 186.0 |  | 0.1641 |  |  |  |  |  |
|  | 0 | 28 |  |  |  |  |  |  |  | 5.07 |  |  | 6.76 |
|  | 1 | 07 | 188.0 |  | 216.0 |  |  |  |  | 5.10 |  |  |  |
|  | 1 | 23 |  | 124.6 |  | 190.4 |  | 0.0916 |  |  |  |  |  |
|  | 1 | 55 |  | 122.8 |  |  |  |  |  |  |  |  |  |
|  | 2 | 23 |  |  |  |  |  |  | 61.0 |  |  |  |  |
|  | 3 | 04 | 188.2 |  | 203.0 |  |  |  |  | 5.09 |  |  |  |
|  | 3 | 23 |  | 125.0 |  | 179.5 |  | 0.1767 |  |  |  |  |  |
|  | 5 | 05 |  | 133.6 |  | 185.0 |  | 0.1431 | 60.0 |  |  |  |  |
| 8 | 5 | 41 | 182.7 |  | 205.8 |  |  |  |  | 5.10 |  |  |  |
|  | 5 | 46 |  | 121.5 |  | 171.0 |  | 0.1662 | 52.0 |  |  |  |  |
|  | 6 | 38 |  |  |  |  |  |  |  |  |  |  |  |
|  | 6 | 51 | 181.0 |  | 231.0 |  |  |  |  | 5.09 |  |  |  |
|  | 6 | 55 |  |  |  |  |  |  | 44.0 |  | 21.0 | 6.0 |  |
|  | 7 | 08 |  |  |  |  |  | 0.0842 |  |  |  |  |  |
|  | 7 | 24 |  | 125.1 |  | 194.2 |  |  |  |  |  |  |  |
|  | 8 | 38 |  |  |  |  |  |  | 44.0 |  |  |  |  |
| Steady State |  |  | 185.0 | 126.0 | 214.0 | 187.0 | - | 0.1377 | 52.2 | 5.09 | 21.0 | 6.0 | 6.76 |

# APPENDIX XV

## DERIVATIONS OF EQUATIONS FOR SUBSTRATE UTILIZATION BY BATCH CULTURES

1. Single substrate utilized for growth and energy of maintenance

$$\frac{dX_\theta^o}{d\theta} = \frac{k^m X_\theta^s}{K+X_\theta^s} X_\theta^o \qquad (212)$$

From substrate balance,

$$-dX_\theta^s = \frac{dX_\theta^o}{Y} + mX_\theta^o d\theta \qquad (213)$$

(a) When K is very small

$$\frac{dX_\theta^o}{d\theta} = k^m X_\theta^o \qquad (214)$$

and

$$X_\theta^o = X_o^o e^{k^m \theta} \qquad (215)$$

From Equations 213, 214, and 215

$$-\frac{dX_\theta^s}{d\theta} = \left[\frac{k^m}{Y} + m\right] X_o^o e^{k^m \theta} \qquad (216)$$

Solution of Equation 216 is

$$X_\theta^o = X_o^s - \frac{X_o^o(k^m+mY)}{Yk^m}\left[e^{k^m\theta}-1\right] \tag{217}$$

(b) When K is large

From Equations 212 and 213

$$-\frac{dX_\theta^o}{d\theta} = \frac{k^m X_\theta^s X_\theta^o + mX_\theta^o Y(K+X_\theta^s)}{Y(K+X_\theta^s)} \tag{218}$$

If $X_o^o$ is much larger than $X_o^s$ so that $X_\theta^o \approx X_o^o$, the solution to Equation 218 is

$$\theta = \frac{Y}{X_o^o(k^m+mY)}\left[(X_o^s-X_\theta^s) + \frac{K\{m-(k^m+mY)\}}{k^m+mY}\ln\left\{\frac{mYK+(k^m+mY)X_\theta^s}{mYK+(k^m+mY)X_\theta^s}\right\}\right] \tag{219}$$

2. Utilization of glucose from a mixture of glucose and galactose

If glucose supplies the growth requirement and galactose supplies the energy requirement, then

$$-dX_\theta^g = U_g' dX_\theta^o \tag{220}$$

and

$$-\frac{dX_\theta^g}{d\theta} = U_g' X_\theta^o \frac{k_g^m X_\theta^g}{K+X_\theta^g} \tag{221}$$

From mass balance

$$X_\theta^o = X_o^o + (X_o^g-X_\theta^g)/U_g' \tag{222}$$

Substituting Equation 222 into Equation 221

$$-\frac{dX_\theta^g}{d\theta} = k_g^m \frac{X_\theta^g(X_o^o U_g' + X_o^g - X_\theta^g)}{K_g + X_\theta^g} \tag{223}$$

Solution of Equation 223 is

$$\ln X_\theta^g = \ln \left[\frac{X_o^o U_g' + X_o^g - X_\theta^g}{U_g'}\right]\left[\frac{X_o^g}{X_o^o}\right] + \frac{X_o^o U_g' + X_o^g}{K_g} \ln \frac{X_o^o U_g' + X_o^g - X_\theta^g}{X_o^o U_g'} - \frac{k_g^m \theta}{K_g} (X_o^o U_g' + X_o^g \tag{224}$$

Equations 217, 219, and 224 are identical with Equations 169, 172, and 170, respectively, of Chapter VIII.

## LITERATURE CITED

1. Thenard, L. J., "Memoire sur la Fermentation Vineuse," *Annales de Chimie*, *Tome Quarante-Sixieme*, 294-320 (1803).

2. Pasteur, M. L., "Memoire sur la Fermentation Appellée Lactique," *Annales de Chimie et de Physique*, Troisiême Série, *Tome L II*, 404-418 (1858).

3. Pasteur, M. L., "Memoire sur la Fermentation Alcoolique," *Annales de Chimie et de Physique*, Troisiême Série, Tome L VIII, 323-426 (1860).

4. Beijerinck, M. W., *Verzamelde Geschriften*, Vols. 1-5, den Haag, M. Nijhof, Netherlands (1921).

5. Winogradsky, S., *Microbiologie du Sol. Oeuvres Complétes*, Mason et Cie, Paris (1949).

6. Phelps, E. B., "The Disinfection of Sewage and Sewage Filter Effluents," *U. S. Geological Survey Water Supply Paper*, No. 229, 91 pages (1909).

7. Monod, J., "*Recherches sur la Croissance des Cultures Bactériennes*," Herman et Cie, Paris (1942).

8. Snyder, W. M., and Stall, J. B., "Men, Models and Machines in Hydrologic Analysis," *Journal of the Hydraulic Division*, *Proceedings of the ASCE*, Vol. 91, HY2, 85-99 (1965).

9. Gaden, E. L., Jr., "Fermentation Process Kinetics," *Journal of Biochemical and Microbiological Technology and Engineering*, Vol. 1, No. 4, 413-430 (1959).

10. Aiba, S., Humphrey, A. E., and Millis, N. F., *Biochemical Engineering*, Academic Press, New York (1965).

11. Lampen, J. O., "Secretion of Enzymes by Microorganisms," *Fifteenth Symposium of the Society for General Microbiology*, 115-133, Cambridge University Press (April, 1965).

12. Gunsalus, I. C., and Stanier, R. Y., *The Bacteria, A Treatise on Structure and Function, Vol. IV: The Physiology of Growth*, Academic Press, New York (1962).

LITERATURE CITED (Continued)

13. Lamanna, C., and Mallette, M. F., Basic Bacteriology, The Williams and Wilkins Co., Baltimore (1965).

14. Rickenberg, H. V., Cohen, G. N., Buttin, G., and Monod, J., "La Galactoside-Permease d' Escherichi coli," Annales de l' Institut Pasteur, 91, 829-857 (1956).

15. Monod, J., "Mechanism of Enzyme Induction," in Enzymes: Units of Biological Structure and Function, 7-28, Gaebler, O. H., Editor, Academic Press, New York (1956).

16. Pardee, A. B., "An Inducible Mechanism for Accumulation of Melibiose in Escherichia coli," Journal of Bacteriology, 73, 376-385 (1957).

17. Retman, B. J., "Regulation of Enzymatic Activity in the Intact Cell: The β-D-Galactosidase of Escherichi coli," Journal of Bacteriology, 76, 1-14 (1958).

18. Sistrom, W. R., "On the Physical State of the Intracellularly Accumulated Substrates of β-Galactoside-Permease in Escherichi coli," Biochimica et Biophysica Acta, 29, 5, 579-587 (1958).

19. Kopes, A., "Etudes Cinetiques sur la Galactoside-Permease D'Escherichia coli," Biochimica et Biophysica Acta, 40, 70-84 (1960).

20. Horecker, B. L., Thomas, J., and Monod, J., "Galactose Transport in Escherichia coli I. General Properties as Studied in a Galactokinaseless Mutant," Journal of Biological Chemistry, 235, 6, 1580-1585 (1960).

21. Horecker, B. L., Thomas, J., and Monod, J., "Galactose Transport in Escherichia coli II. Characteristic of the Exit Process," Journal of Biological Chemistry, 235, 6, 1586-1590 (1960).

22. Osborn, M. J., McLellan, W. L., and Horecker, B. L., "Galactose Transport in Escherichi coli III. The Effect of 2, 4-Dinitrophenol on Entry and Accumulation," Journal of Biological Chemistry, 236, 10, 2585-2589 (October 1961).

23. Hoffee, P., Englesberg, E., and Lamy, F., "The Glucose Permease System in Bacteria," Biochimica et Biophysica Acta, 79, 337-350 (1964).

24. Yasuhiro, A., "Transport of Sugars and Amino Acids in Bacteria I. Purification and Specificity of the Glucose and Leucine Bonding Proteins," Journal of Biological Chemistry, 243, 11, 3116-3122 (1968).

## LITERATURE CITED (Continued)

25. Jacob, F., and Monod, J., "Genetic Regulatory Mechanisms in the Synthesis of Proteins," Journal of Molecular Biology, 3, 318-356 (1961).

26. Magasanik, B., "Nutrition of Bacteria and Fungi," in Annual Review of Microbiology, Vol. 11, 221-252 (1957).

27. Lumry, R., "Some Aspects of the Thermodynamics and Mechanisms of Enzymic Catalysis," in The Enzymes, Boyer, P., Lardy, H. and Myrback, K., Editors, Vol. 1, Academic Press Inc., New York (1959).

28. Webb, F. C., Biochemical Engineering, D. van Nostrand Co. Ltd., New York (1964).

29. Dixon, M., and Webb, E. C., Enzymes, Academic Press Inc., New York (1964).

30. Karlson, P., Introduction to Modern Biochemistry, Academic Press, New York and London (1965).

31. Davis, B. D., "The Teleonomic Significance of Biosynthetic Control Mechanisms," Cold Spring Harbor Symposium on Quantitative Biology, Vol. XXVI, 1-10 (1961).

32. Cheldelin, V. H., "Metabolic Pathways in Microorganisms," E. R. Squibb Lectures on Chemistry of Microbial Products, John Wiley & Sons, Inc., New York (1960).

33. Knaysi, G., "Elements of Bacterial Cytology," 2nd Edition, Cornell University Press, Ithaca, N. Y. (1951).

34. Monod, J., "The Phenomenon of Enzymatic Adaptation and its Bearing on Problems of Genetics and Cellular Differentiation," Growth, Vol. XI, No. 1, 223-289 (March 1947).

35. Powell, E. O., "An Outline of the Pattern of Bacterial Generation Times," Journal of General Microbiology, 18, 382-417 (1958).

36. Kubitschek, H. E., "Normal Distribution of Cell Generation Rates," Nature, 209, 5027, 1039-1040 (March 1966).

37. Painter, P. R., and Marr, A. G., "Inequality of Mean Interdivision Time and Doubling Time," Journal of General Microbiology, 48, 155-159 (1967).

38. Monod, J., "The Growth of Bacterial Cultures," Annual Review of Microbiology, 3, 371-394 (1949).

LITERATURE CITED (Continued)

39. Bail, O., "Ergebnisse Experimenteller Populationsforshung," Zitä tsf. Immun. Forsch., 60, 1-22 (1929).

40. Dawes, E. A., and Ribbons, D. W., "Some Aspects of the Endogenous Metabolism of Bacteria," Bacteriological Reviews, 28, 2, 126-149 (June 1964).

41. Strange, R. E., Dark, F. A., and Ness, A. G., "The Survival of Stationary Phase Aerobacter aerogenes, Stored in Aqueous Suspensions," Journal of General Microbiology, 25, 61-76 (1961).

42. Postgate, J. R., and Hunter, J. R., "The Survival of Starved Bacteria," Journal of General Microbiology, 29, 233-263 (1962).

43. Encylopedia Americana, International Edition, Vol. 18, page 171, Americana Corporation, New York (1965).

44. Enclopedia Britannica, Vol. 14, page 717, Encyclopedia Britannica Inc., William Benton (Publisher), Chicago (1967).

45. Verhulst, P. F., "Recherches Mathematiques sur la loi d' aecroissement de la Population," Memoires de l' Academic Royale des Sciences des Lettres et des Beaux-Arts de Belgique, Bruxelles, XVIII (1945), XX (1947).

46. Pearl, R., and Reed, L. J., "On The Rate of Growth of the Population of the United States Since 1790 and its Mathematical Representation," Proceeding of the National Academy of Sciences, 6, 6, 275-288 (1920).

47. Keshevan, K., Behn, V. C., and Ames, W. F., "Kinetics of Aerobic Removal of Organic Wastes," Journal of the Sanitary Engineering Division, ASCE, 90, SA1, 99-126 (February 1964).

48. Edwards, V. H., and Wilke, C. R., "Mathematical Representation of Batch Culture Data," Biotechnology and Bioengineering, Vol. X, 205-232 (1968).

49. Brody, S., "Bioenergeties and Growth, with Special Reference to the Efficiency Complex in Domestic Animals," Hafner Publishing Co., New York (1964).

50. Thompson, D. A. W., "On Growth and Form," Cambridge University Press, Cambridge (1944).

51. Beran, K., Discussion part of the Symposium on the Continuous Cultivation of Microorganisms held in Prague in June 1958, Folica Microbiologica, 4, 390 (1959).

## LITERATURE CITED (Continued)

52. Dean, A. C. R., and Hinshelwood, C., *Growth, Function andRegulation in Bacterial Cells*, Oxford University Press (1966).

53. Harrison, A. P., Jr., "The Response of Bacterium *Lactis aerogenes* when Held at Growth Temperature in the Absence of Nutrient: An Analysis of Survival Curves," *Proceedings of the Royal Society* (London), *Series B*, *152*, 418-428 (1952).

54. Burleigh, I. G., and Dawes, E. A., "Studies on the Endogenous Metabolism and Senescence of Starved *Sarcina lutea*," *Biochemical Journal*, *102*, 236-250 (1967).

55. Campbell, A., "Synchronization of Cell Division," *Bacteriological Reviews*, *21*, 263-272 (1957).

56. Slator, A., "The Rate of Growth of Bacteria," *Journal of Chemical Society*, *109*, 2-10 (1916); *Chemical Abstract*, *10*, 11 (June 1916).

57. Kono, T., "Kinetics of Microbial Cell Growth," *Biotecynology and Bioengineering*, *Vol. X*, 105-131 (1968).

58. Powell, E. O., "Theory of the Chemostat," *Laboratory Practice*, *14*, 81 (Oct. 1965).

59. Herbert, D., "Some Principles of Continuous Culture," *VII International Congress for Microbiology*, *Recent Progress in Microbiology*, Tunevall, G. (Editor), Almquist and Wiksell, Stockholm, 381-402 (1958).

60. Powell, E. O., "Discussion on Continuous Culture Methods and Their Application, *Symposium VI at International Congress for Microbiology at Stockholm*, *Recent Progress in Microbiology*, Tunevall, G. (Editor), Stockholm (1959).

61. Downing, A. L., and Wheatland, A. B., "Fundamental Considerations in Biological Treatment of Effluents," *Transactions of the Institution of Chemical Engineers*, *40*, 91-103 (1962).

62. Lipe, R. S., "The Relationship between Substrate Concentration, Respiration Rate and Growth Rate of *Escherichia coli* in Continuous Flow Cultures," Ph.D. Dissertation, *Dissertation Abstract*, *22*, 1796-1797 (1961).

63. Schulze, K. L., and Lipe, R. S., "Relationship between Substrate Concentration, Growth Rate, and Respiration Rate of *Escherichia coli* in Continuous Culture," *Archiv für Mikrobiologie*, *48*, 1-20 (1964).

## LITERATURE CITED (Continued)

64. Schulze, K. L., "The Activated Sludge Process as a Continuous Flow Culture," Water and Sewage Works, Vol. 111, No. 12, 526-538 (December 1964).

65. Schulze, K. L., "The Activated Sludge Process as a Continuous Flow Culture," Water and Sewage Works, Vol. 112, No. 1, 11-17 (Jan. 1965).

66. Marr, A. G., Nilson, E. H., and Clark, D. J., "The Maintenance Requirement of Escherichia coli," Annals of New York Academy of Sciences, 102, 536-548 (1963).

67. Hempfling, W. P., and Vishniac, W., "Yield Coefficients of Thiobacillus neapolitanus in Continuous Culture," Journal of Bacteriology, 93, 3, 874-878 (March 1967).

68. Pirt, S. J., "The Maintenance Energy of Bacteria in Growing Culture," Proceedings of the Royal Society, Series B, 163, 224-231 (1965).

69. Mallette, M. F., "Validity of the Concept of Energy of Maintenance," Annals of the New York Academy of Sciences, 102, 3, 521-535 (January 1963).

70. McGrew, S. B., and Mallette, M. F., "Energy of Maintenance in Escherichia coli," Journal of Bacteriology, 83, 844-850 (1962).

71. Rich, L. G., Unit Processes of Sanitary Engineering, John Wiley & Sons, Inc., New York and London (1963).

72. Teissier, G., "Les Lois Quantitatives de la Croissance," Annales de Physiologie et de Physicochimie Biologique, 12, 527 (1936).

73. Novick, A., and Szilard, L., "Experiments with the Chemostat on Spontaneous Mutations of Bacteria," Proceedings of the National Academy of Science, 36, 708-719 (1950).

74. Novick, A., "Experimentation with the Chemostat," Recent Progress in Microbiology, G. Tunevall (Editor), 403-415 (1959).

75. Spicer, C. C., "The Theory of Bacterial Growth Apparatus," Biometrics, 11, 225-230 (June 1955).

76. Moser, H., "Structure and Dynamics of Bacterial Population Maintenance in the Chemostat," Cold Spring Harbor Symposium of Quantitative Biology, Vol. XXII, Cold Spring Harbor, Long Island, N.Y., 121-137 (1957).

LITERATURE CITED (Continued)

77. Moser, H., "The Dynamics of Bacterial Populations Maintained in Chemostat," Carnegie Institute Publication No. 614, Carnegie Institution, Washington, D. C. (1958).

78. Ierusalimsky, N. D., "The Conditions of Growth of Microorganisms in Continuous Flow Cultures," in Continuous Cultivation of Microorganisms: A Symposium, 53, Publishing House of the Czechoslovak Academy of Science, Prague (1958).

79. Contois, D. E., "Kinetics of Bacterial Growth: Relationship Between Population Density and Specific Growth Rate of Continuous Culture," Journal of General Microbiology, 21, 40-50 (1959).

80. van Uden, N., "Transport Limited Growth in the Chemostat and its Competitive Inhibition; A Theoretical Treatment," Archiv für Mikrobiologie, 58, 145-154 (1967).

81. Saidel, G. M., "Bacterial Cell Populations in a Continuously Changing Environment," Journal of Theoretical Biology, 19, 287-296 (1968).

82. Leudeking, R., and Piret, E. L., "Transient and Steady States in Continuous Fermentation: Theory and Experiment," Journal of Biochemical and Microbiological Technology and Engineering, 1, 431-459 (1959).

83. Schulze, K. L., "The Effect of Phosphate Supply on the Rate of Growth and Fat Formation in Yeasts," Applied Microbiology, Vol. 14, 207-210 (March 1956).

84. Butterfield, C. T., "Experimental Studies of Natural Purification in Polluted Waters, III. A Note on the Relation Between Food Concentration in Liquid Media and Bacterial Growth," Public Health Reports, 44, 2865-2872 (1929).

85. Butterfield, C. T., Purdy, W. C., and Theriault, E. J., "Experimental Studies of Natural Purification in Polluted Waters, IV. The Influence of the Plankton on the Biochemical Oxidation of Organic Matter," Public Health Reports, 46, 393-461 (1931).

86. Fair, G. M., and Moore, E. W., "Mathematical Formulation of the Course of Digestion," Sewage Works Journal, 4, 3, 428-443 (1932).

87. Garrett, M. J., and Sawyer, C. N., "Kinetics of Removal of Soluble BOD by Activated Sludge," Proceedings 7th Industrial Waste Conference, Purdue University Extension Service, 79, 51-77 (1952).

88. Chynoweth, D. P., "The Effect of Glucose on the Anaerobic Sludge Fermentation," ESE Notes, School of Public Health, University of North Carolina, 6, 1, 1-2 (January 1969).

## LITERATURE CITED (Continued)

89. Garrett, T. M., "Kinetics of the Removal of Soluble Substrate by Activated Sludge," Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge (1951).

90. McCabe, Brother J., "Mathematical Formulation of the Biological Oxidation Process," Air and Water Pollution--An International Journal, 5, 265-276, Pergamon Press, Oxford (1963).

91. Wilson, I. S., "Concentration Effects in the Biological Oxidation of Trade Wastes," International Journal of Air and Water Pollution, 7, 99-114, Pergamon Press, Oxford (1963).

92. Gram, A. L., "Reaction Kinetics of Aerobic Biological Processes," Report No. 2, I.E.R. Series 90, University of California, Berkeley, California (1956).

93. McKinney, R. E., Symons, J. M., Shifrin, G., and Vezina, M., "Design and Operation of a Completely Mixed Activated Sludge System," Sewage and Industrial Wastes, 30, 3, 287-295 (1958).

94. McKinney, R. E., "Mathematics of Complete Mixing Activated Sludge," Journal of the Sanitary Engineering Division, ASCE, 88, SA3, 87-113 (May 1962).

95. Gates, W. E., Smith, J. H., Lin, Shun-Dar, and Ris III, C. H., "An Anaerobic-Aerobic Process for Industrial Wastewater Treatment, I. The Anaerobic Process," Presented at the National Meeting of the Water Pollution Control Federation, Atlantic City, N. J. (October 1965).

96. Andrews, J. F., and Pearson, E. A., "Kinetics and Characteristics of Volatile Acid Production in Anaerobic Fermentation Processes," International Journal of Air and Water Pollution, 9, 439-461, Pergamon Press, Oxford (1965).

97. Kornegay, B. H., and Andrews, J. F., "Kinetics of Fixed Film Biological Reactors," Journal of the Water Pollution Control Federation, 40, 11, Part 2 R460-R468 (November 1968).

98. Kornegay, B. H., and Andrews, J. F., "Kinetics of Fixed Film Biological Reactors," 22nd Annual Purdue Industrial Waste Conference, Purdue University, Lafayette, Indiana (May, 1967).

99. Jenkins, D., and Garrison, W. E., "Control of Activated Sludge by Mean Cell Residence Time," Journal of the Water Pollution Control Federation, 40, 11, Part 1, 1905-1919 (November, 1968).

100. Stratton, F. E., and McCarty, P. C., "Prediction of Nitrification Effects on Dissolved Oxygen Balance of Streams," Environmental Science and Technology, 1, 5, 405-410 (May 1967).

LITERATURE CITED (Continued)

101. Brown, A. J., "Enzyme Action," Journal of the Chemical Society (London), Paper No. XXXVI, Vol. LXXXI, 373-388 (1902).

102. Henri, V., "Lois Generales de L'action des Diatases," These, Hermann, Paris (1903).

103. Michaelis, L., and Menten, M. L., "Die Kinetik der Invertinwiekung," Biochenische zeitschrift, 49, 333-369 (1913).

104. Briggs, G. E., and Haldane, J. B. S., "A Note on the Kinetics of Enzyme Action," Biochemical Journal, Vol. XIX, No. 2, 338-339 (1925).

105. Knowles, G., Downing, A. L., and Barrett, M. J., "Determination of Kinetic Constants for Nitrifying Bacteria in Mixed Culture, with the Aid of an Electronic Computer," Journal of General Microbiology, 38, 263-278 (1965).

106. Gates, N. E., and Marlar, J. T., "Graphical Analysis of Batch Culture Data Using the Monod Expressions," Journal Water Pollution Control Federation, 40, 11, Part 2, R469-R476 (1968).

107. Deans, H. A., "Kinetics on the Microbial Scale," Rice University Studies, 52, 2, 43-52 (1966).

108. Christensen, H. N., and Palmer, G. A., Enzyme Kinetics, W. B. Saunders Company, Philadelphia and London (1967).

109. Bauchop, T., and Elsden, S. R., "The Growth of Microorganisms in Relation to Their Energy Supply," Journal of General Microbiology, 23, 457-469 (1960).

110. Hetling, L. J., Washington, D. R., and Rao, S. S., "Kinetics of the Steady State Bacterial Culture, II. Variation in Synthesis," Air and Water Pollution: International Journal, Vol. 10, 357-383, Pergamon Press, Oxford (1966).

111. Schroeder, A. M., and Busch, A. W., "Validity of Energy Change as a Growth Parameter," Journal of the Sanitary Engineering Division, ASCE, Vol. 94, SA2, 193-200 (April 1968).

112. Servizi, J. A., and Bogan, R. H., "Free Energy as a Parameter in Biological Treatment," Journal of the Sanitary Engineering Division, ASCE, Vol. 89, SA3, 17-40 (June 1963).

113. McCarty, P. L., "Thermodynamics of Biological Synthesis and Growth," Paper presented at the 2nd International Conference on Water Pollution Research, Tokyo, Japan (August 1960).

## LITERATURE CITED (Continued)

114. McKinney, R. E., Microbiology for Sanitary Engineers, McGraw-Hill Book Co., Inc., New York, 1962.

115. McKinney, R. E., McCarty, P. L., and Hernandez, J. W., Discussion of "Free Energy as a Parameter in Biological Treatment," by Servizi, J. A., and Bogan, R. H., Journal of the Sanitary Engineering Division, ASCE, 89, 1, 61 (December 1963).

116. Plack, O. R., and Rochhoft, C. C., "Studies of Sewage Purification, XVII. The Utilization of Organic Substrate by Activated Sludge," Sewage Works Journal, 19, 3, 423-440 (1947).

117. Sawyer, C. N., "Bacterial Nutrition and Synthesis, Biological Treatment of Sewage and Industrial Waste, Vol. I, 3-17, Reinhold Publishing Corporation, New York (1956).

118. McCabe, B. J., and Eckenfelder, W. W., "Process Design of Biological Oxidation for Industrial Waste Treatment; Waste Treatment, Pergamon Press, Oxford (1960).

119. Helmers, E. N., Frame, J. D., Greenberg, A. E., and Sawyer, C. N., "Nutritional Requirements in the Biological Stabilization of Industrial Wastes--II. Treatment with Domestic Sewage," Sewage and Industrial Waste, 23, 7, 884-899 (1951).

120. Henkelekian, H., Orferd, H. E., and Manganelli, R., "Factors Affecting the Quantity of Sludge Production in the Activated Sludge Process," Sewage and Industrial Waste 23, 8, 945-958 (1951).

121. Hoover, S. R., Jasewicz, L., Pepinsky, J. B., and Porges, N., "Assimilation of Dairy Wastes by Activated Sludge," Sewage and Industrial Waste, 23, 2, 167-173 (1951).

122. Dienert, F., "Sur la Fermentation du Galactose," Annales Institut Pasteur, 14, 139-189 (1900).

123. Stephenson, M., and Yudkin, J., "Galactozymase Considered as an Adaptive Enzyme," Biochemical Journal, 30, 506-514 (1936).

124. Monod, J., "Sur un Phénoméne Nouveau de Croissance Complexe dans les Cultures Bactériennes," Academie des Sciences, Paris, Comptes Rendus Hebdomadaires des Séances 212, 934-936 (1941).

125. Karström, H., "Uber die Enzymbildung in Bakterien und Uber Einige Physiologische Eigenschaften der Untersuchten Bakterienarten," Thése Helsingfors (1930).

126. Karström, H., "Enzymatische Adaptation bei Mikroorganismen," Ergebnisse der Enzymforschung, 7, 350-376 (1938).

## LITERATURE CITED (Continued)

127. Epps, H. M. R., and Gale, E. F., "The Influence of the Presence of Glucose During Growth on the Enzymic Activities of Escherichia coli: Comparison of the Effect with That Produced by Fermentation Acids," Biochemical Journal, 36, 619-623 (1942).

128. Spiegelman, S., and Dunn, R., "Interactions Between Enzyme-Forming Systems During Adaptation," Journal of General Physiology, 31, 153-173 (1947).

128a. Gale, E. F., "Factors Influencing the Enzymic Activities of Bacteria," Bacteriological Reviews, 7, 3, 139-173 (1943).

129. Ravin, A. W., "Discontinuous Variability in the Utilization of Carbon Sources by Aerobacter aerogenes," Ph.D. Dissertation, Columbia University, New York (1951).

130. Ravin, A. W., "Heritable and Non-heritable Loss of Ability by Aerobacter aerogenes to Grow Adaptively on Single Carbon Sources," Journal of General Microbiology, 6, 211-232 (1952).

131. Dagley, S., and Dawes, E. A., "Citric Acid Metabolism of Aerobacter aerogenes," Journal of Bacteriology, 66, 259-265 (1953).

132. Davis, B. D., "Units of Biological Structure and Function," pages 509-522 in Enzymes, Gaebler, O. H. (Editor), Academic Press, Inc., New York (1956).

133. Boezi, J. A., and deMoss, R. D., "Properties of a Tryptophan Transport System in Escherichia coli," Biochimica et Biophysica Acta, 49, 471-484 (1961).

134. Baidya, T. K. N., Webb, F. C., and Lilly, M. D., "The Utilization of Mixed Sugars in Continuous Fermentation. I.," Biotechnology and Bioengineering, Vol. IX, 195-204 (1967).

135. Harte, M. J., and Webb, F. C., "Utilization of Mixed Sugars in Continuous Fermentation. II.," Biotechnology and Bioengineering, Vol. IX, 205-221 (1967).

136. Hosler, P., and Jhonson, M. J., "Penicillin from Chemically Defined Media," Industrial and Engineering Chemistry, 45, 4, 871-874 (1953).

137. Magasanik, B., "The Metabolic Control of Histidine Assimilation and Dissimilation in Aerobacter aerogenes," Journal of Biological Chemistry, 213, 557-569 (1955).

## LITERATURE CITED (Continued)

138. Neidhardt, F. C., and Magasanik, B., "The Effect of Glucose on the Induced Biosynthesis of Bacterial Enzymes in the Presence and Absence of Inducing Agents," *Biochimica et Biophysica Acta*, *21*, 324-334 (1956).

139. Hamilton, W. A., and Dawes, E. A., "A Diauxic Effect with *Pseudomonas aeruginosa*," *Biochemical Journal*, *71*, 25p-26p (1959).

140. Hamilton, W. A., and Dawes, E. A., "The Nature of the Diauxic Effect with Glucose and Organic Acids in *Pseudomonas aeruginosa*," *Biochemical Journal*, *76*, 70p (1960).

141. Magasanik, A. K., and Bojarska, A., "Enzyme Induction and Repression by Glucose in *Aerobacter aerogenes*," *Biochemical and Biophysical Research Communications*, *2*, 17-81 (1960).

142. Neidhardt, F. C., "Mutant of *Aerobacter aerogenes* Lacking Glucose Repression," *Journal of Bacteriology*, *80*, 536-543 (1960).

143. Pardee, A. B., "Response of Enzyme Synthesis and Activity to Environment," *Eleventh Symposium of the Society for General Microbiology*, London, 19-40, Cambridge University Press (April 1961).

144. Stanier, R. Y., "Adaptation, Evolutionary and Physiological: Or Darwinism Among the Micro-organisms," *Third Symposium of the Society for General Microbiology*, London, 1-14, Cambridge University Press (April 1953).

145. Duguid, J. P., and Wilkinson, J. F., "Environmentally Induced Changes in Bacterial Morphology," *Eleventh Symposium of the Society for General Microbiology*, London, 69-99, Cambridge University Press (April 1961).

146. Fisher, K. W., "Environmental Influence on Genetic Recombination in Bacteria and Their Viruses," *Eleventh Symposium of the Society for General Microbiology*, London, 272-295, Cambridge University Press (April 1961).

147. Murrell, W. G., "Spore Formation and Germination as a Microbial Reaction to the Environment," *Eleventh Symposium of the Society for General Microbiology*, London, 100-150, Cambridge University Press (April 1961).

148. Ravin, A. W., "The Nature of Variation Affecting Bacterial Adaptability," *Third Symposium of the Society for General Microbiology*, London, 46-69, Cambridge University Press (April 1953).

149. Spiegelman, S., "Modern Aspects of Enzymatic Adaptation," in *The Enzymes*, *1*, Part 1, 267-306, Academic Press, New York (1951).

## LITERATURE CITED (Continued)

150. Monod, J., and Cohn, M., "La Biosynthèse Induite des Enzymes," *Advances in Enzymology*, *13*, 67-119 (1952).

151. van Niel, C. B., "Natural Selection in the Microbial World," *The Second Marjory Stephenson Memorial Lecture*, *Journal of General Microbiology*, *13*, 1, 201-217 (April 1955).

152. Cohn, M., and Monod, J., "Specific Inhibition and Induction of Enzyme Biosynthesis," *Third Symposium of the Society for General Microbiology*, 132-149, Cambridge University Press (April 1953).

153. Pollock, M. R., "Stages in Enzyme Adaptation," *Third Symposium of the Society for General Microbiology*, London, 150-177, Cambridge University Press (April 1953).

153a. Spiegelman, S., and Halverson, H. O., "The Nature of the Precurser in the Induced Synthesis of Enzymes," *Third Symposium of the Society for General Microbiology*, London, 98-125, Cambridge University Press (April 1953).

154. Monod, J., and Cohn, M., "Sur le Mécanisme de la Synthèse d'une Proteine Bacterienne," Symposium on Microbial Metabolism, *Sixth International Congress of Microbiology*, Rome, 42-62 (1953).

155. Rotman, B., and Spiegelman, G., "On the Origin of the Carbon in the Induced Synthesis of β-Galactosidase in *Escherichia* *coli*," *Journal of Bacteriology*, *68*, 419-429 (1954).

156. Spiegelman, S., Reiner, J. M., and Cohnberg, R., "The Relation of Enzymatic Adaptation to the Metabolism of Endogenous and Exogenous Substrates," *Journal of General Physiology*, *31*, 27-49 (1947).

157. Monod, J., "Inhibition de l'adaptation Enzymatique chez *B.* *Coli* en Presence de 2-4-Dinitrophenol," *Annales Institut Pasteur*, *70*, 381-384 (1944).

158. Spiegelman, S., "Nuclear and Cytoplasmic Factors Controlling Enzymatic Constitution," *Cold Spring Harbor Symposium on Quantitative Biology*, Cold Spring Harbor, New York, *11*, 256-277 (1946).

159. Sussman, M., and Spiegelman, S., "The Effect of Arsenate and Azide on the Stability of Adaptive Enzymes," *Archives of Biochemistry*, *29*, 54-68 (1950).

160. Spiegelman. S., "The Dissociation of Anaerobic Metabolism from Enzymatic Adaptation in Yeast," *Journal of Cellular Comparative Physiology*, *30*. 315-329 (1947).

## LITERATUVE CITED (Continued)

161. Stanier, R. Y., "Enzymatic Adaptation in Bacteria," Annual Review of Microbiology, 5, 35-56 (1951).

162. Knox, R., "The Effect of Temperature on Enzymic Adaptation, Growth and Drug Resistance," Third Symposium of Society for General Microbiology, London, 184-195, Cambridge University Press (April 1953).

163. Magasanik, B., "Catabolite Repression," Cold Spring Harbor Symposium on Quantitative Biology, Vol. XXVI, 249-254 (1961).

164. Axelrod, B., "Glycolysis" and "Other Pathways of Carbohydrate Metabolism," in Metabolic Pathways, Greenberg, D. M., Editor, Vol. 1, 112-146 and 309-393, Academic Press, New York and London (1967).

165. Clarke, P. H., and Meadow, P. M., "Evidence of the Occurrence of Permeases for Tricarboxylic Acid Cycle Intermediates in Pseudomonas aeruginosa," Journal of General Microbiology, 20, 144-155 (1959).

166. Jacob, F., and Monod, J., "On the Regulation of Gene Activity," Cold Spring Harbor Symposium on Quantitative Biology, Vol. XXVI, Long Island, New York, 193-209 (1961).

167. Anonymous, "Genetics: Turned-off Genes," Time, 51 (April 28, 1967).

168. Ames, B. N., and Hartman, P. E., "The Histidine Operon," Cold Spring Harbor Symposium on Quantitative Biology, 28, 349-356 (1963).

169. Nass, W., and McFall, E., "Genetic Aspects of Metabolic Control," Annual Review of Microbiology, 18, 95-110 (1964).

170. Stent, G. S., "The Operon: On Its Third Anniversary," Science, 144, 816-820 (1964).

171. Brenner, C., and Beckwith, J. R., "Ochre Mutants, a New Class of Suppressible Nonsense Mutants," Journal of Molecular Biology, 13, 629-637 (1965).

172. Brenner, G., "Theories of Gene Regulation," British Medical Bulletin (London) 21, 244-248 (1965).

173. Beckwith, J. R., "Regulation of the Lac Operon," Science, 156, 597-604 (May 1967).

174. Epstein, W., and Beckwith, J. R., "Regulation of Gene Expression," Annual Review of Biochemistry, 37, 411-436 (1968).

LITERATURE CITED (Continued)

175. Sadler, J. R., and Novick, A., "The Properties of Repressor and the Kinetics of its Action," *Journal of Molecular Biology*, *12*, 305-327 (1965).

176. Gelbert, W., and Müller-Hill, B., "Isolation of the Lac Repressor," *Proceedings of the National Academy of Science*, U.S., *56*, 1891-1898 (1966).

177. Hayashi, M., Spiegelman, S., Franklin, N., and Luria, S. E., "Separation of the RNA Message Transcribed in Response to a Specific Inducer," *Proceedings of the National Academy of Science*, Washington, D. C., *49*, 729-736 (1968).

178. Hartwell, L. H., and Magasanik, B., "The Mechanism of Histidase Induction and Formation in *Bacillus subtilis*," *Journal of Molecular Biology*, *10*, 105-119 (1964).

179. Neidhardt, F. C., and Magasanik, B., "Inhibitory Effect of Glucose on Enzyme Formation," *Nature*, *178*, 801-802 (1956).

180. Neidhardt, F. C., and Magasanik, B., "Reversal of the Glucose Inhibition of Histidase Biosynthesis in *Aerobacter aerogenes*," *Journal of Bacteriology*, *73*, 253-259 (1957).

181. Ames, B. N., and Martin, R. G., "Biochemical Aspects of Genetics: The Operon," *Annual Review of Biochemistry*, *33*, 235-258 (1964).

182. McFall, E., and Mandelstam, J., "Specific Metabolic Repression of Induced Enzymes in *Escherichia coli*," *Nature*, *197*, 4870,880-881 (March 1963).

183. Gorini, L., "Regulation en Retour (Feedback Control) de la Synthese de l'Arginine chez *Escherichia Coli*," *Bulletin Société de Chimie Biologique* (Paris), *40*, 1939-1952 (1958).

184. Nakada, D., and Magasanik, B., "The Roles of Inducer and Catabolite Repressor in the Synthesis of β-Galactosidase by *Escherichia coli*," *Journal of Molecular Biology*, *8*, 105-127 (1964).

185. Loomis, W. F., and Magasanik, B., "The Catabolite Repression Gene of the *Lac* Operon in *Escherichia coli*," *Journal of Molecular Biology*, *23*, 487-494 (1967).

186. Lardy, H. A., Potter, R. L., and Burris, R. H., "Metabolic Functions of Biotin. I. The Role of Biotin in Bicarbonate Utilization by *Lactobacillus arabinosus* Studied with $C^{14}$," *Journal of Biological Chemistry*, *179*, 721-731 (1949).

LITERATURE CITED (Continued)

187. Monod, J., and Cohen-Bazire, G., "L'effet d'Inhibition Specifique dans la Biosynthese de la Tryptophane-Desmase chez *Aerobacter aerogenes*," *Comptes Rendus* Hebdomadaires des Sèances de l'Academie des Sciences, *236*, 530-532 (1953).

188. Cohn, M., Cohen, G., and Monod, J., "L'effet Inhibiteur Spècifique de la Mèthionine dans la Formation de la Mèthionine Synthase chez *Escherichia coli*," *Comptes Rendus* Hebdomadaires des Sèances de l'Academie des Sciences, *236*, 746-648 (1953).

189. Adelberg, E. A., and Umbarger, H. E., "Isoleucine and Valine Metabolism in *Escherichia coli*," *Journal of Biological Chemistry*, *205*, 475-482 (1953).

190. Wijesundera, S., and Woods, D. D., "The Effect of Growth on a Medium Containing Methionine on the Synthesis of this Amino Acid by *Bacterium coli*," *Biochemical Journal*, *55*, viii (1953).

191. Novick, A., and Szilard, L., "Experiments with the Chemostat on the Rates of Amino Acid Synthesis in Bacteria," in *Dynamics of Growth Processes*, Boell, E. J., Editor, 21, Princeton University Press, N. J. (1954).

192. Vogel, H., in *The Chemical Basis of Heredity*, McElroy, W. D., and Glass, B., Editors, 276, Johns Hopkins Press, Baltimore, Maryland (1956).

193. Gorini, L., and Maas, W. K., "The Potential for the Formation of a Biosynthetic Enzyme in *Escherichia coli*," *Biochimica et Biophysica Acta*, *25*, 208-209 (1947).

194. Vogel, H. J., "Repressed and Induced Enzyme Formation: A Unified Hypothesis," *Proceedings of the National Academy of Science*, Washington, D. C., *43*, 491-496 (1957).

195. Roberts, R. B., Abelson, P. H., Bolton, E. T., and Britten, R. J., "Studies of Biosynthesis in *Escherichia coli*," *Publication of the Carnegie Institution, No. 607* (1957).

196. Yates, R., and Pardee, A. B., "Control of Enzyme Formation by Products," *Journal of Biological Chemistry*, *227*,2,677-692 (1957).

197. Magasanik, B., Magasanik, A. K., and Neidhardt, F. C., "Regulation of Growth and Composition of the Bacterial Cell," 334-349, in *Regulation of Cell Metabolism*, Wolstenholme and O'Connor, Editors, Little, Brown and Company, Boston (1959).

198. Novick, A., "Growth of Bacteria," *Annual Review of Microbiology*, *9*, 97-110 (1955).

## LITERATURE CITED (Continued)

199. Pardee, A. B., Shore, V. G., and Prestidge, L. S., "Incorporation of Azatryptophan into Proteins, Bacteria and Bacteriophage," *Biochimica et Biophysica Acta*, *21*, 406-407 (1956).

200. Muneer, R., and Cohen, G. N., "Incorporation d'analogues Structureaux d'Amino-acids dans les Proteins Bactèriennes," *Biochimica et Biophysica Acta*, *21*, 592-593 (1956).

201. Misra, U. K., and French Dexter, "Inhibition and Action Pattern of β-Amylase in Presence of Maltose," *396th Meeting of the Biochemical Society held at the University of Bristol* (July 1960).

202. Halvorson, H., and Ellias, L., "The Purification and Properties of an α-Glucosidase of *Saccharomy italicus* Y 1225," *Biochimica et Biophysica Acta*, *30*, 28-40 (1958).

203. Haldane, J. B. S., *Enzymes*, The M.I.T. Press, Massachusetts Institute of Technology, Cambridge, Massachusetts, Series MIT 36 (1964).

204. Kennell, D., and Magasanik, B., "The Control of the Rate of Enzyme Synthesis in *Aerobacter aerogenes*," *Biochimica et Biophysica Acta*, *81*, 418-434 (1964).

205. Kornberg, H. L., "The Co-ordination of Metabolic Routes," *Fifteenth Symposium of the Society for General Microbiology*, London, Cambridge University Press (April 1965).

206. Cline, A. L., and Bock, R. M., "Translational Control of Gene Expression," *Cold Spring Harbor Symposium on Quantitative Biology*, *31*, 321-333 (1966).

207. McFall, E., and Mass, W. K., "Regulation of Enzyme Synthesis in Microorganisms," in *Molecular Genetics*, *2*, 255-321, Taylor, J. H., Editor, Academic Press, New York (1967).

208. Benzer, S., "Induced Synthesis of Enzymes in Bacteria Analyzed at the Cellular Level," *Biochimica et Biophysica Acta*, *11*, 383-395 (1953).

209. Adler, J., "Chemotaxis in Bacteria," *Science*, *153*, 708-715 (August 1966).

210. Hulcher, F. H., Young, R. W., and Kendall, W. K., "Sparing of Cellulose by Amino Acids Among Aerobic Soil Bacteria," *Journal of Bacteriology*, *73*, 703-705 (1957).

211. Gaudy, A. F., "Studies on Induction and Repression in Activated Sludge Systems," *Applied Microbiology*, *10*, 264-271 (1962).

## LITERATURE CITED (Continued)

212. Gaudy, A. F., Jr., Gaudy, E. T., and Komolrit, K., "Multicomponent Substrate Utilization by Natural Populations and a Pure Culture of *Escherichia coli*," *Applied Microbiology*, *11*, 157-162 (1963).

213. Gaudy, A. F., Komolrit, K., and Bhatla, M. N., "Sequential Substrate Removal in Heterogeneous Populations," *Journal of the Water Pollution Control Federation*, *35*, 7, 903-922 (1963).

214. Bhatla, M. N., and Gaudy, A. F., "Sequential Substrate Removal in Dilute Systems by Heterogeneous Microbial Population," *Applied Microbiology*, *13*, 8, 345-347 (1965).

215. Stumm-Zollinger, E., "Inhibition and Repression in Substrate Utilization," *Progress Report WP 00621*, Engineering Science Laboratory, Harvard University, Cambridge, Mass. (August 1965).

216. Stumm-Zollinger, E., "Effects of Inhibition and Repression on the Utilization of Substrates by Heterogeneous Bacterial Communities," *Applied Microbiology*, *14*, 4, 654-664 (July 1966).

217. Stumm-Zollinger, E., "Substrate Utilization in Heterogeneous Bacterial Communities," *Journal of the Water Pollution Control Federation*, *40*, 5, Part 2, R213-R229 (1968).

218. Stumm-Zollinger, E., Busch, P. L., and Stumm, W., Discussion of "Kinetics of Aerobic Removal of Organic Wastes" by Keshavan, K., Behn, B. C., and Ames, W. E., *Journal of the Sanitary Engineering Division*, ASCE, SA4, 107-113 (1964).

219. Hernandez, J. W., Jr., "Aerobic Bacterial Uptake of Dissolved Nutrients Between 10 and 70°C," *Ph.D. Dissertation*, Harvard University, Cambridge, Massachusetts (June 1965).

220. Gates, W. E., Mancy, K. H., Shafie, F. R., and Pohland, F. G., "A Simplified Physical Model for Studying Assimilative Capacity," *21st Industrial Waste Conference*, Purdue University, Extension Service, Lafayette, Indiana (May 1966).

221. Marlar, J. T., "The Effect of Turbulence on Bacterial Substrate Utilization," *M.S. Thesis*, School of Civil Engineering, Georgia Institute of Technology, Atlanta, Georgia (December 1967).

222. Chian, G. K., and Mateles, R. I., "Growth of Mixed Cultures on Mixed Substrates. I. Continuous Culture," *Journal of Applied Microbiology*, *10*, 1337-1342 (1968).

223. Mateles, R. I., Chian, S. K., and Silver, R. S., "Continuous Culture on Mixed Substrates," in *Microbial Physiology and Continuous Culture*, Powell, E. O., Evans, C. G. T., Strange, R. E., and Tempest, D. W., Editors, Her Majesty's Stationary Office, London (1967).

## LITERATURE CITED (Continued)

224. Mateles, R. I., "Kinetics of Uptake in Pure and Mixed Cultures," *Abstract of Papers*, *American Chemical Society 154th Meeting* (1967).

225. Jannasch, H. W., "Growth of Marine Bacteria at Limiting Concentrations of Organic Carbon in Seawater," *Limnology and Oceanography*, *12*, 264-271 (1967).

226. Jannasch, H. W., "Continuous Culture in Microbial Ecology," *Laboratory Practice*, *14-83*, 1162-1167 (October 1965).

227. Monod, J., "La Technique de Culture Continue Theorie et Applications," *Annales Institut Pasteur*, *79*, 390-410 (1950).

228. Novick, A., and Szilard, L., "Description of Chemostat," *Science*, *112*, 715-716 (1950).

229. Novick, A., and Szilard, L., "Genetic Mechanisms in Bacteria and Bacterial Viruses. I. Experiments on Spontaneous and Chemically Induced Mutations of Bacteria Growing in the Chemostat," *Cold Spring Harbor Symposium on Quantitative Biology*, *16*, 337-343 (1951).

230. Herbert, D., "The Chemical Composition of Micro-organisms as a Function of Their Environment," *Eleventh Symposium of the Society for General Microbiology*, Cambridge University Press, 391-416 (April 1961).

231. Dean, A. C. R., and Hinshelwood, C., "Variation in Nucleic Acid Content of *Bact. lactis aerogenes*," *Proceedings Royal Society of London*, *Series B*, *151*, 944, 348-363 (February 1960).

232. Dean, A. C. R., "Nucleic Acid and Protein Content of *Bact. lactis aerogenes*. I. Equilibrium and Non-equilibrium Conditions," *Proceedings Royal Society of London*, *Series B*, *155*, 961, 580-588 (April 1962).

233. Dean, A. C. R., "Variation During Conditions of Nutrient Deficiency," *Proc. Roy. Soc., London*, *Series B*, *155*, 961, 588-598 (April 1962).

234. Holme, T., "Continuous Culture Studies on Glycogen Synthesis in *Escherichia coli*," *Acta Chimica Scandinavica*, *11*, 763-775 (1957).

235. Pirt, S. J., "The Oxygen Requirements of Growing Cultures of an *Aerobacter* species Determined by Means of the Continuous Culture Technique," *Journal of General Microbiology*, *16*, 59-75 (1957).

236. Mor, J. R., and Fiechter, A., "Continuous Cultivation of *Saccharomyces cerevisiae*. I. Growth on Ethanol under Steady State Conditions," *Biotechnology and Bioengineering*, Vol. X, 2, 159-176 (1968).

## LITERATURE CITED (Continued)

237. Powell, E. O., "Criteria for the Growth of Contaminants and Mutants in Continuous Culture," Journal of General Microbiology, 18, 259-268 (1958).

238. Bryson, V., "The Turbidostatic Selection--A Device for Automatic Isolation of Bacterial Variants," Science, 116, 48-51 (1952).

239. Jannasch, H. W., "Enrichments of Aquatic Bacteria in Continuous Culture," Archiv für Mikrobiologie, 59, 165-173 (1967).

240. Herbert, D., Elsworth, R., and Telling, R. C., "The Continuous Culture of Bacteria; a Theoretical and Experimental Study," Journal of General Microbiology, 14, 601-622 (1956).

241. Martin, E. J., and Washington, D. R., "Kinetics of the Steady State Bacterial Culture. I. Mathematical Model," Air and Water Pollution International Journal, 10, 343-355, Pergamon Press, Oxford (1966).

242. Maxon, W. D., "A Microbiological Process Report: Continuous Fermentation: A Discussion of its Principles and Applications," Applied Microbiology, 3, 2, 110-122 (May 1955).

243. Gerhardt, P., and Bartlett, N. C., "Continuous Industrial Fermentations," Advances in Applied Microbiology, 1, 215-260, Academic Press, New York (1959).

244. James, T. W., "Continuous Culture of Microorganism," Annual Review of Microbiology, 15, 27-46, Annual Reviews, Inc., Palo Alto, California (1961).

245. Malek, I., and Hsopedka, J., "Continuous Cultivation of Microorganism: A Review," Folia Microbiologica, 5, 120-139 (1960).

246. Malek, I., and Fencl, Z., "Continuous Cultivation of Microorganism: A Review," Folia Microbiologica, 6, 192-208 (1961).

247. Malek, I., and Beran, K., "Continuous Cultivation of Microorganisms: A Review," Folia Microbiologica, 7, 388-411 (1962).

248. Greenhalgh, R. E., Johnson, R. L., and Nott, H. D., "Mixing in Continuous Reactors," Chemical Engineering Progress, 55, 2, 44-48 (February 1959).

249. Herbert, D., "A Theoretical Analysis of Continuous Culture Systems," Society of Chemical Industries Monograph No. 12, 21-53 (1961).

250. Grieves, R. B., and Kao, R., "Input and Volume Distributions for Continuous Cultures," Biotechnology and Bioengineering, Vol. X, 497-510 (1968).

## LITERATURE CITED (Continued)

251. Bryson, V., "The Turbidostatic Selector--A Device for Automatic Isolation of Bacterial Variants," Science, 116, 48 (1952).

252. Bryson, V., "Application of Continuous Culture to Microbial Selection," Recent Progress in Microbiology, Almquist and Wiksell, Tunevall, G., Editor, Stockholm (1959).

253. Bungay, H. R., and Bungay, M. L., "Microbial Interactions in Continuous Culture," Manuscript submitted to Advances in Applied Microbiology (December 1967).

254. Kornberg, H. L., "The Metabolism of $C_2$ Compounds in Microorganisms, 1. The Incorporations of $2\text{-}C^{14}$ Acetate by Pseudomonas fluorescens and Corynebacterium grown on Ammonium Acetate," Biochemical Journal, 68, 535-542 (1958).

255. Herbert, D., "Continuous Culture of Microorganisms," Proceedings of the 2nd Symposium 1962, Chzechoslovak Academy of Science, 121-131 (1964).

256. Mateles, R. I., Ryu, D. Y., and Yasuda, T., "Measurement of Unsteady State Growth Rates of Microorganisms," Nature, 208, 263-265 (1965).

257. Humphrey, A. E., "Some Observations of Continuous Fermentation," Proceedings 5th Symposium, Institute of Applied Microbiology, University of Tokyo, 215 (1963).

258. Grieves, R. B., Milbury, W. F., and Pipes, W. O., "The Effect of Short-Circuiting upon the Completely-Mixed Activated Sludge Process," International Journal of Air and Water Pollution, 8, 199-214, Pergamon Press (1964).

259. Milbury, W. F., and Pipes, W. O., "A Laboratory Study of the Effects of Shortcircuiting Upon the Completely-Mixed Activated Sludge Process," International Journal of Air and Water Pollution, 9, 41-53, Pergamon Press (1965).

260. Herbert, D., "Continuous Culture of Microorganisms: Some Theoretical Aspects," in Continuous Cultivation of Microorganisms, A Symposium, 45-52, Publishing House of the Ceskoslovenska Akademie Věd, Prague (1958).

261. Button, D. K., and Garver, J. C., "Continuous Culture of Torulopois utilis: A Kinetic Study of Oxygen Limited Growth," Journal of General Microbiology, 45, 195-204 (1966).

262. Duclaux, E., Traite de Microbiologie, 1, 208, Masson, Paris (1898).

## LITERATURE CITED (Continued)

263. Bartsch, A. F., and Ingram, W. M., "Stream Life and the Pollution Environment," *Public Works*, 104-110 (July 1959).

264. Brock, T. D., "*Principles of Microbial Ecology*," Prentice Hall, Inc., Englewood Cliffs, New Jersey (1966).

265. Renneboog-Squilbin, C., "Theory of Chemostat--Study of the Conditions under which a Population of Micro-organisms and its Mutant Will Coexist in the Chemostat," *Journal of Theoretical Biology*, **14**, 74-101 (1967).

266. Dias, F. F., and Bhat, J. V., "Microbial Ecology of Activated Sludge. I. Dominant Bacteria," *Applied Microbiology*, **12**, 5, 412-417 (September 1964).

267. Prakasam, T. B. S., and Dondero, N. C., "Aerobic Heterotrophic Bacterial Populations of Sewage and Activated Sludge," *Applied Microbiology*, **15**, 1128-1137 (1967).

268. Collard, P. J., and Gossling, J., "Continuous Culture of Mixed Population," *Proceedings for Society for General Microbiology*, 50th General Meeting, Churchill, Cambridge (September 1967).

269. Bower, G., and Gaddis, L., "Filamentous Waste Treatment Systems at Low pH," Paper presented before the 41st *Annual Conference of Water Pollution Control Federation*, Chicago (September 1968).

270. Cassell, E. A., Sulzer, F. T., and Lamb, III, J. C., "Population Dynamics and Selection in Continuous Mixed Cultures," *Journal Water Pollution Control Federation*, **38**, 9, 1398-1409 (1966).

271. Kalckar, H. M., "Uridine Diphospho Galactose: Metabolism, Enzymology and Biology," *Advances in Enzymology*, Nord, F. F., Editor, **20**, 111-134 (1958).

272. Wu, H. C. P., "Role of the Galactose Transport System in the Establishment of Endogenous Induction of the Galactose Operon in *Escherichia coli*," *Journal of Molecular Biology*, **24**, 213-223 (1967).

273. Wu, H. C. P., and Kalckar, H. M., "Endogenous Induction of the Galactose Operon in *Escherichia coli* K12*," *Proceedings of the National Academy of Science*, Washington, **55**, 623-629 (1966).

274. Rotman, B., and Radojkovic, J., "Galactose Transport in *Escherichia coli*," *Journal Biological Chemistry*, **239**, 11, 3153-3156 (October 1964).

## LITERATURE CITED (Continued)

275. Britten, R. J., "The Concentration of Small Molecules Within the Microbial Cell," *Fifteenth Symposium of the Society for General Microbiology*," Cambridge University Press (April 1965).

276. Yasuhiro, A., "Transport of Sugars and Amino Acids in Bacteria. II. Properties of Galactose and Leucine-Binding Proteins," *Journal of Biological Chemistry*, 243, 11, 3123-3127 (1968).

277. Kepes, A., "Etudes Cinétiques sur la Galactoside-Permease de *E. coli*," Abstracts of the IVth *International Congress of Biochemistry*, Vienna, 1958, p. 78, Pergamon Press, Ltd., London (1958).

278. Trucco, R. E., Caputto, R., Leloir, L. F., and Mittelman, N., "Galactokinase," *Archives of Biochemistry*. 18, 137-146 (1948).

279. Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E., and Paladini, A., "Isolation of the Coenzyme of the Galactose Phosphate-Glucose Phosphate Transformation," *Journal of Biological Chemistry*, 184, 333 (1950).

280. Leloir, L. F., "The Enzymatic Transformation of Uridine Diphosphate Glucose into a Galactose Derivative," *Archives of Biochemistry and Biophysics*, 33, 186-190 (1951).

281. Trucco, R. E., "Enzymatic Synthesis of Uridine Diphosphate Glucose," *Archives of Biochemistry and Biophysics*, 34, 482-483 (1951).

282. Kalckar, H. M., "Biochemical Mutations in Man and Microorganisms," *Science*, 125, 105-108 (1957).

283. Kalckar, H. M., Braganca, B., Munch-Peterson, A., "Uridyl Transferases and the Formation of Uridine Diphosphogalactose," *Nature*, 172, 1038 (1953).

284. Kurahashi, K., "Enzyme Formation in Galactose-Negative Mutants of *Escherichia coli*," *Science*, 125, 114-116 (1957).

285. Leloir, L. F., "The Metabolism of Hexosephosphates," 67-93, in *Phosphorus Metabolism*, Vol. 2, McElroy, W. D., and Glass, B., Editors, The Johns Hopkins Press, Baltimore (1951).

286. Heidelberger, M., Barker, S. A., and Stacey, M., "Components of the Specific Polysaccharides of Types IX, XII, and XIV *Pneumococcus*," *Science*, 120, 781-782 (1954).

287. Salton, N. R., "Studies of the Bacterial Cell Wall," *Biochimica et Biophysica Acta*, 10, 512-523 (1953).

## LITERATUVE CITED (Continued)

288. Cardini, C. E., "The Hexokinases of *Escherichia coli*," *Enzymologia*, *14*, 362-368 (1951).

289. Kalckar, H. M., Kurahashi, K., and Jordan, E., "Hereditary Defects in Galactose Metabolism in *Escherichia coli* Mutants, I. Determination of Enzyme Activities," *Proceedings of National Academy of Sciences*, U. S., *45*, 1776-1786 (1959).

290. Kurahashi, K., and Sugimura, A., "Purification and Properties of Galactose-1-Phosphate Uridyl Transferase from *Escherichia coli*," *Journal of Biological Chemistry*, *235*, 940-946 (1960).

291. Buttin, G., "Some Aspects of Regulation in the Synthesis of the Enzymes Governing Galactose Metabolism," *Cold Spring Harbor Symposia on Quantitative Biology*, Long Island Biological Association, Cold Spring Harbor, New York, 26, 213-216 (1961).

292. Kowalsky, A., and Koshland, D. E., Jr., "The Mechanism of the Galactose-Glucose Interconversion in *Lactobacillus bulgaricus*," *Biochimica et Biophysica Acta*, *22*, 575-577 (1956).

293. Rutter, W. J., and Hansen, R. G., "Lactose Metabolism, I. Carbohydrate Metabolism of *Lactobacillus bulgaricus* Strain Gere A*," *Journal of Biological Chemistry*, *202*, 311-321 (1953).

294. Fukasawa, T., and Nikaido, H., "Galactose Mutants of *Salmonella typhimurium*," *Genetics*, *46*, 1295-1303 (1961).

295. Fukasawa, T., and Nikaido, H., "Galactose-Sensitive Mutants of Salmonella, II. Bacteriolysis Induced by Galactose," *Biochimica et Biophysica Acta*, *48*, 470-483 (1961).

296. Kosterlitz, H. W., "Galactose-1-Phosphate in the Liver During Galactose Assimilation," *Journal of Physiology* (London), *93*, 34P-35P (1938).

297. Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E., and Paladini, A., "A Coenzyme for Phospho-glucomutase," *Archives of Biochemistry*, *18*, 201-203 (1949).

298. Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., "Isolation of the Coenzyme of the Galactose Phosphate-Glucose Phosphate Transformation," *Journal of Biological Chemistry*, *184*, 333-350 (1950).

299. Sunderajan, T. A., Rapin, A. M. C., and Kalckar, H. M., "Biochemical Observations on *E. Coli* Mutants Defective in Uridine Diphosphoglucose," *Proceedings of the National Academy of Sciences* (Washington), *48*, 2187-2193 (1962).

## LITERATURE CITED (Continued)

300. Maxwell, E. S., "The Enzymic Interconversion of Uridine Diphosphogalactose and Uridine Diphosphoglucose," *Journal of Biological Chemistry*, *229*, 139-151 (November 1957).

301. Wilson, D. B., and Hogness, D., "The Enzymes of the Galactose Operon in *Escherichia coli*. I. Purification and Characterization of Uridine Diphosphogalactose 4-Epimerase," *Journal of Biological Chemistry*, *239*, 8, 2469-2481 (August 1964).

302. Wilson, D. B., and Hogness, D., "Galactokinase and Uridine-Diphosphogalactose-4-Epimerase from *Escherichia coli*," in *Methods in Enzymology*, Neufeld, E. F., and Ginsberg, V., Editors, Vol. VIII, 229-240, Academic Press, New York (1960).

303. Hansen, R. G., and Craine, E. M., "Lactose Metabolism, III. The Reversible Conversion of Galactose-1-Phosphate to Glucose-1-Phosphate," *Journal of Biological Chemistry*, *208*, 293-298 (1954).

304. Shigeru, S., Ozutsumi, M., and Kurahashi, K., "Galactose-1-Phosphate Uridyltransferase of *Escherichia coli* II. Further Purification and Characterization," *Journal of Biological Chemistry*, *242*, 10, 2362-2368 (May 1967).

305. Heinrich, M. R., "The Purification and Properties of Yeast Galactokinase," *Journal of Biological Chemistry*, *239*, 1, 50-53 (January 1964).

306. Sherman, J. R., and Adler, J., "Galactokinase from *Escherichia* coli," *Journal of Biological Chemistry*, *238*, 3. 873-878 (March 1963).

307. DeLey, J., and Dondoroff, N., "The Metabolism of D-Galactose in *Pseudomonas saccharophila*," *The Journal of Biological Chemistry*, *227*, 2, 745-757 (August 1957).

308. Buttin, G., "Mècanismes Règulateurs dans la Biosynthèse des Enzymes du Mètabolisme du Galactose chez *Escherichia coli* K12. II. Le Dèterminisme Gènetique de la Règulation," *Journal of Molecular Biology*, *7*, 183-205 (1963).

309. Buttin, G., "Mècanismes Regulateurs dans la Biosynthèse des Enzymes du Mètabolisme du Galactose chez *Escherichia coli* K12. I. Biosynthèse Induite de la Galactokinase et l'Induction Simultanee du la Sèquence Enzymatique," *Journal of the Molecular Biology*, *7*, 164-182 (1963).

310. Hearon, J. Z., "Rate Behavior of Metabolic System," *Physiological Reviews*, *32*, 499-523 (1952).

## LITERATURE CITED (Continued)

311. Krebs, H. A., and Kornberg, H. L., "Energy Transformations in Living Matter," Berlin: Springer-Verlag (1957).

312. Burton, A. C., "The Basis of the Principle of the Master Reaction in Biology," *Journal Cellular and Comparative Physiology*, *9*, 1-14 (1936).

313. Proper, G., and Garver, J. C., "Mass Culture of the Protozoa *Colopoda steinii*," *Biotechnology and Bioengineering*, *8*, 287-296 (1966).

314. Schroepfer, G. J., Fullen, W. J., Johnson, A. S., Ziemke, N. R., and Anderson, J. J., "The Anaerobic Contact Process as Applied to Packinghouse Wastes," *Sewage and Industrial Wastes*, *27*, 4, 460-486 (1955).

315. Schroepfer, G. J., and Ziemke, N. R., "Development of the Anaerobic Contact Process. I. Pilot-Plant Investigations and Economics," *Sewage and Industrial Wastes*, *31*, 2, 164-190 (1959).

316. Scnichiji, S., and Uematsu, T., "Continuous Cultivation and Fermentation of Lactic Acid Bacteria. (2) On the Death of *Lactobacillus acidophilus* during Fermentation and its Determination," *Kogyo Gijutsuin Hakko Kenkyu Hokoku*, *24*, 259-274 (1963).

317. Taub, F. B., and Dollar, A. M., "Improvement of a Continuous-Culture Apparatus for Long-term Use," *Applied Microbiology*, *16*, 2, 232-235 (February 1968).

318. Elsworth, R., Meakin, L. R. P., Pirt, S. J., and Capell, G. H., "A Two Litre Scale Continuous Culture Apparatus for Microorganisms," *Journal of Applied Bacteriology*, *19*, 2, 264-278 (1956).

319. Perret, C. J., "An Apparatus for the Continuous Culture of Bacteria at Constant Population Density," *Journal of General Microbiology*, *16*, 250-264 (1957).

320. Herbert, D., Phipps, P. J., and Tempest, D. W., "The Chemostat: Design and Instrumentation," *Laboratory Practice*, 14-82 (October 1965).

321. DeHaan, P. G., and Winkler, K. C., "An Apparatus for the Continuous Culture of Bacteria at Constant Generation Times," „*Nederlandsche Tijdschrift Voor Hygiene*, *Microbiologie en Serologie*" (Amsterdam), *21*, 33-44 (1955).

322. Parker, R. B., "Gravity Flow System for Continuous Culture Feed," *Applied Microbiology*, *15*, 1, 197 (January 1967).

## LITERATURE CITED (Continued)

323. Mancy, K. H., and Westgarth, W. C., "A Galvanic Cell Oxygen Analyzer," *Journal of Water Pollution Control Federation*, *34*, 10, 1037-1051 (1962).

324. Callow, D. S., and Pirt, S. J., "Automatic Control of pH Value in Cultures of Microorganisms," *Journal of General Microbiology*, *14*, 661-671 (1956).

325. Lovelock, H. R., and Nicholas, D. J. D., "Growth of *Nitrosomonas europaea* in Batch and Continuous Culture," *Archiv für Mikrobiologie*, *61*, 302-309 (1968).

326. Tempest, D. W., Dicks, J. W., and Hunter, J. R., "The Interrelationship between Potassium, Magnesium and Phosphorus in Potassium Limited Chemostat Culture," *Journal of General Microbiology*, *45*, 135-146 (1966).

327. *Standard Methods for the Examination of Water and Waste Water*, 12th Edition, American Public Health Association, Inc., New York (1965).

328. Eckenfelder, W. W., and O'Connor, D. J., *Biological Waste Treatment*, Pergamon Press, New York (1961).

329. Powell, E. O., "A Rapid Method of Determining the Properties of Viable Bacteria in a Culture," *Journal of General Microbiology*, *14*, 153-159 (1956).

330. Fung, D. Y. C., and Kraft, A. A., "Microtiter Method for the Evaluation of Viable Cells in Bacterial Cultures," *Applied Microbiology*, *16*, 7, 1036-1039 (July 1968).

331. Gaden, E. L., "Aeration and Oxygen Transport in Biological Systems--Basic Considerations," in *Biological Treatment of Sewage and Industrial Wastes*, *Volume 1* (Editors: McCabe, B. J., and Eckenfelder, W. W.,) Reinhold Publishing Corporation, New York (1956).

332. Bronzes, P., "Control of Respiratory Metabolism of Activated Sludge and the Automatic Working of Purification Plants Based on This Measurement," *Water Research*, *2*, 1, 56-58 (January 1968).

333. Hattingh, W. H. J., "Respiration Studies on Activated Nitrifying and Biological Filter Humus Sludges, Part 2. Respiration Rate and Sludge Concentration," *The Water and Waste Treatment Journal*, *7*, 12, 538-542 (1960).

334. Spiegelman, S., "The Physiology and Genetic Significance of Enzymatic Adaptation," *Annals of Missouri Botanical Garden*, St. Louis, *32*, 139-163 (1945).

## LITERATURE CITED (Continued)

335. Spiegelman, S., Reiner, J., and Cohnberg, R., "The Relation of Enzymatic Adaptation to the Metabolism of Endogenous and Exogenous Substrates," Journal of General Physiology, 21, 27-49, (1947).

336. Pollock, M. R., "The Influence of Temperature on the Adaptation of Tetrathionase in Washed Suspensions of Bact. Paratyphosum B.," British Journal of Experimental Pathology, 26, 410-416 (1945).

337. Pollock, M. R., "Adaptation of "Nitratase" in Washed Suspension of Bacteria," British Journal Experimental Pathology, 27, 419-432 (1946).

338. Yale, E. F., "The Bacterial Amino Acid Decarboxylases," Advances in Enzymology, 6, 1-32 (1946).

339. Rose, I. A., "Acetate Kinase of Bacteria (Acetokinase)." Methods in Enzymology, Vol. I, (Editors: Colowick, S. P., and Kaplan, N. O.) Academic Press Inc., New York (1955).

340. Lotze', J. P., "Methods for the Determination of Intermediary Enzymes in Mixed Cultures Used for the Purification of Organic Polluted Waters," Water Research, Vol. 1, 351-365, Pergamon Press (1967).

341. Hershey, A. D. and Bronfenbrennar, J., "Factors Limiting Bacterial Growth. III. Cell Size and 'Physiologic Youth' in Bacterium coli Cultures," Journal of General Physiology, 21, 721-728 (1937-38).

342. Monod, J., Pappenheimer, A. M., and Cohen-Bazire, G., "La Cinétique de la Biosynthése de la β-Galactosidase chiz E. coli Considéree comme Fonction de la Croissance," Biochimica et Biophysica Acta, 9, 648-660 (1952).

343. Cohn, M., "Contribution of Studies on the β-Galactosidase of Escherichia coli to Our Understanding of Enzyme Synthesis," Bacteriological Reviews, 21, 140-168 (1957).

344. Warburg, O., Wasserstoffiibertragende Fermente, Dr. W. Saenger (Publisher), Berlin (1948).

345. Bergmeyer, H. U., Methods of Enzymatic Analysis, Academic Press, New York (1963).

346. Pechman, H. V.. and Runge, P., "Oxydation der Formazylverbindungen," Berichte der Deutschen Chemischen Gesellschaft, 27, 2920-2930 (1894).

## LITERATURE CITED (Continued)

347. Kuhn, R., and Jerchel, D., "Uber Invertseifen. VII. Tetrazalium-salze, VIII. Reduktion von Tetrazoliumsalzen durch Bakterien, Gärende Hefe und Keimende Samen," Berichte der Deutschen Chemischen Gasellschaft, 74, 941-952 (1941).

348. Mattson, A. M., Jensen, C. O., and Dutcher, R. A., "Triphenyltetrazolium Chloride as a Dye for Vital Tissues," Science, 106, 294-295 (Sept., 1947).

349. Jensen, C. O., Sacks, W., and Baldauske, F. A., "The Reduction of Triphenyltetrazolium Chloride by Dehydrogenases of Corn Embryos," Science, Vol. 113, 65-66 (1951).

350. Jerchel, D., and Möhle, W., "Die Bestimmung des Reduktionspotentials von Tetrazoliumverbindungen," Berichte der Deutschen Chemischen Gesellschaft, 77, 8, 591-601 (1944).

351. Lenhard, G., "Dehydrogenase Activity of the Soil as a Measure of Microbial Activity in the Soil," Zeitschrift für Pflanzenernähr Düngung und Bodenkunde, 73, 1-11 (1956).

352. Bucksteeg, W., and Thiele, H., "Verbesserung der Keimzählungstechnik in der Wasserbakteriologie durch Anwendung von 2-3-5-Triphenyltetrazoliumchlorid (TTC)," Zentralblatt für Bakteriologie Parasitenkunde, Infektionskrankheilen und Hygiene, II. Abt., 111, 5235 (1958).

353. Bucksteeg, W., and Thiele, H., "Die Beurteilung von Abwasser und Schlamm Mittels TTC (2-3-5-Triphenyltetrazoliumchloride)," Das Gas- und Wasserfach, 100, 916-920 (1959).

354. Lenhard, G., "Methods for the Examination of Refuse and Refuse Sludge Compost," Paper presented at the International Conference for the Disposal of Domestic and Industrial Waste, Essen (May 1962).

355. Lenhard, G., Ross, W., and Du Plooy, A., "A Study of Methods for the Classification of Bottom Deposits of Natural Waters," Hydrobiologia, 20, 223-240 (1962).

356. Lenhard, G., and Du Plooy, A., "A New Method for the Determination of the Anionic Adsorption Capacity of Bottom Deposits with Particular Reference to the Adsorption of Anionic Synthetic Detergents," Hydrobiologia, 21, 177-187 (1963).

357. Lenhard, G., "Studies on the Adsorption and Biochemical Degradation of Synthetic Detergents in Bottom Deposits," Hydrobiologia, 22, 3-4, 323 (1963).

## LITERATURE CITED (Continued)

358. Bucksteeg, W., and Thiele, H., "Beziehung Zwischen Biochemisher Abbauleistung und Reduktasenaktivität von Belebtschlamm," Das Gas-und Wasserfach, 105, 1278-1292 (1964).

359. Lenhard, G., Nourse, L. D., and Schwartz, H. M., "The Measurement of Dehydrogenase Activity of Activated Sludges," Advances in Water Pollution Research, Vol. 2, Second International Conference held in Tokyo, 105-119, Pergamon Press (August 1964).

360. Lenhard, G., "The Dehydrogenase Activity as a Criterion for the Determination of Toxic Effects in Biological Purification Systems," Hydrobiologia, 25, 1-8 (1965).

361. Lenhard, G., "Bottom Deposits--A Vital Self-Purification System in the Biological Degradation of Waste in Natural Waters and Biological Treatment of Effluents," Hydrobiologia, 25, 3-4, 411 (1965).

362. Bucksteeg, W., "Determination of Sludge Activity--A Possibility of Controlling Activated Sludge Plants," Paper presented at the Third International Conference on Water Pollution, Munich (September 1966).

363. Ford, D. L., Yang, J. T., and Eckenfelder, W. W., "Dehydrogenase Enzyme as a Parameter of Activated Sludge Activities," Proceedings of the 21st Industrial Waste Conference, Purdue University Extension Service, 121, 665-674 (1966).

364. Fred, R. B., and Knight, S. G., "The Reduction of 2,3,5-Triphenyltetrazolium Chloride by Penicillium chrysogenum," Science, 109, 169-170 (1949).

365. Martin, H. H., "Bacterial Protoplasts--A Review," Journal of Theoretical Biology, 5, 1, 1-34 (1963).

366. Malatyan, M. N., and Biryuzova, V. I., "Localization of Dehydrogenase Activity in Mitochondrial Analogs of Bacterial Cells," Proceedings of the Academy of Sciences of the U.S.S.R., Biological Sciences Section, 160 (January-February 1965).

367. Fraser, D., "Bursting Bacteria by Release of Gas Pressure," Nature, 167, 33-34 (January 6, 1951).

368. Parr Instrument Company, "Cell Disruption Bomb," Product Sheet 4635, Parr Instrument Co., Molini, Illinois (June 1968).

369. Millipore Filter Corporation, "Analysis and Control of Contamination in Aviation Fuels," Application Guide AG-1, 9-12 (1966).

## LITERATURE CITED (Continued)

370. Aerospace Recommended Practice, ARP 785, "Procedure for the Determination of Particulate Contamination in Hydraulic Fluids by the Control Filter Gravimetric Procedure," Society of Automotive Engineers, Inc., New York (February 1963).

371. Hald, A., "Statistical Theory with Engineering Applications," 571-584, John Wiley & Sons, London (1962).

372. Somogyi, M., "A New Reagent for the Determination of Sugars," Journal of Biological Chemistry, 160, 61-68 (1945).

373. Shaffer, P. A., and Somogyi, M., "Copper-Iodometric Reagents for Sugar Determination," Journal of Biological Chemistry, 100, 695-713 (1933).

374. Somogyi, M., "A Reagent for the Copper-Iodometric Determination of Very Small Amounts of Sugar," Journal of Biological Chemistry, 117, 771 (1937).

375. Nelson, N., "A Photometric Adaptation of the Somogyi Method for the Determination of Glucose," Journal of Biological Chemistry, 153, 375-380 (1944).

376. Muller, D., "Studien uber ein neues Enzym Glykoseoxydase," Biochem. Z., 199, 136-139 (1928).

377. Keilin, D., and Hartree, E. F., "Properties of Glucose Oxidase (Notatin), Biochemical Journal, 42, 221-225 (1948).

378. Keilin, D., and Hartree, E. F., "Specificity of Glucose Oxidase (Notatin)," Biochemical Journal, 50, 331-332 (1952).

379. Keaton, A. S., "Specific Colorimetric Enzymatic Reagents for Glucose," Proceedings 129th Meeting of the American Chemical Society, 31c (April 1956).

380. Huygett, A. St. G., and Nixon, D. A., "The Determination of Blood Glucose Using Mixed Enzyme-Oxygen Acceptor Reagent," Journal of Biological Chemistry, 66, 12P (1957).

381. Huygett, A. St. G., and Nixon, D. A., "Use of Glucose Oxidase, Peroxidase, and o-dianisidine in Determination of Blood and Urinary Glucose," Lancet, 2, 368-370 (1957).

382. Hill, J. B., and Kessler, G., "An Automated Determination of Glucose Utilizing a Glucose-Oxidase Peroxidase System," Journal of Laboratory and Clinical Medicine, 57, 970-980 (1961).

## LITERATURE CITED (Continued)

383. Discombe, G., "An Inexpensive Method for the Estimation of True Glucose in Blood and Other Fluids by the Autoanalyzer," Journal of Clinical Pathology, 16, 170-172 (1963).

384. Robin, M., and Saifer, A., "Determination of Glucose in Biologic Fluids with an Automated Enzymatic Procedure," Clinical Chemistry, 11, 840-845 (1965).

385. Jeris, J. S., and Cardenas, Jr., R. R., "Glucose Determination in Biological Treatment Systems," Applied Microbiology, 14, 6, 857-864 (1966).

386. Updike, S. J., and Hicks, G. P., "Reagentless Substrate Analysis with Immobilized Enzymes," Science, 158, 270-272 (October 1967).

387. "Glucostat for the Enzymatic Determination of Glucose," Worthinton Biochemical Corporation, Freehold, New Jersey (1965).

388. "Galactostat: A Coupled Enzyme System for the Determination of Galactose," Worthinton Biochemical Corporation, Freehold, New Jersey (1966).

389. Cooper, C. M., Fernstrom, G. A., and Miller, S. A., "Performance of Agitated Gas-Liquid Contactors," Industrial and Engineering Chemistry (Industr.), 36,6,504-509 (1944).

390. MacMullin, R. B., and Weber, M., Jr., "The Theory of Short-Circuiting in Continuous Flow Mixing Vessels in Series and the Kinetics of Chemical Reactions in Such Systems," Transactions of the American Institute of Chemical Engineers, 31, 2, 409-458 (1935).

391. Maxon, W. D., and Johnson, M. J., "Aeration Studies on Metabolism of Bakers Yeast," Industrial and Engineering Chemistry, 45, 11, 2554-2560 (1953).

392. Fair, G. M., and Geyer, J. C., Water Supply and Waste Water Disposal, John Wiley and Sons, Inc. (1954).

393. Butler, J. N., Ionic Equilibrium: A Mathematical Approach, Addison-Wesley Publishing Company, Inc., Reading, Massachusetts (1964).

394. Hoover, S. R., and Porges, N., "Assimilation of Dairy Wastes by Activated Sludge, II. The Equation of Synthesis and Rate of Oxygen Utilization," Sewage and Industrial Wastes, 24, 3, 306-312 (1952).

395. Mor, J. R., and Fiechter, A., "Continuous Cultivation of Saccharomyces corevisiae. II. Growth on Ethanol under Transient-State Conditions," Biotechnology and Bioengineering, Vol. X, 787-803 (1968).

## LITERATURE CITED (Continued)

396. Dias, F. F., Dondero, N. C., and Finstein, M. S., "Attached Growth of Sphaerotilus and Mixed Populations in a Continuous-Flow Apparatus," Applied Microbiology, 16, 8, 1191-1199 (August 1968).

397. Anderson, P. A., "Automatic Recording of the Growth Rates of Continuously Cultured Microorganisms," Journal of General Physiology, 36, 733-737 (1953).

398. Gaudy, A. F., Jr., Ramanathan, M., and Rao, B. S., "Kinetic Behavior of Heterogeneous Populations in Completely Mixed Reactors," Biotechnology and Bioengineering, 9, 387-411 (1967).

399. Bungay, H. R., Abstract, American Chemical Society Meeting, 152nd New York (1966).

400. Contois, D. E., and Yango, L. D., Abstract, American Chemical Society Meeting, 148th, Chicago (1964).

401. Fuld, G. J., Mateles, R. I., and Kusmierck, B. W., "A Method for the Study of the Dynamics of Continuous Fermentation," Society of Chemical Industries Monograph, 12, 54-67 (1961).

402. Rao, K. S., "The Kinetics of Continuous Culture of E. coli," M.S. Thesis, University of Wisconsin, Madison, Wisconsin (1962).

403. Hobson, P. N., "Continuous Culture of Some Anaerobic and Facultatively Anaerobic Rumen Bacteria," Journal of General Microbiology, 30, 167-180 (1965).

404. Kipnis, D. M., Helmreich, E., and Cori, C. F., "Studies of Tissue Permeability, IV. The Distribution of Glucose Between Plasma and Muscle," Journal Biological Chemistry, 234, 165-170 (1959).

405. Kalckar, H. M., Robichon-Szulmajster de Huguette, and Kurahasi, K., "Galactose Metabolism in Mutants of Man and Mikroorganisms," Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 52-56 (1957).

406. Cohen, G. N., and Monod, J., "Bacterial Permeases," Bacteriological Reviews, 79, 169-194 (1957).

407. Jannasch, H. W., "Eine Notiz über die Anreicherung von Mikroorganismen in Chemostaten," Anreicherungskultur und Mutantenauslese Zentr. Bakteriol, Vol. 1 (suppl.), 498-502 (1965).

408. "Tentative Methods of Test for Particulate Contaminant in Aviation Turbine Fuels," D2276-657 (Issued 1964, Revised 1965), Book of ASTM Standards, Part 17, 839-847 (1966).

## LITERATURE CITED (Concluded)

409. Fair, G. M., "The 'Log-Difference' Method of Estimating the Constants of the First-Stage Biochemical Oxygen Demand Curve," Sewage Works Journal, 8, 3, 430-434 (1936).

# VITA

Sambhunath (Sam) Ghosh was born on August 1, 1935, in Calcutta, India, the eldest son of Taraknath and Annapurna Ghosh. He attended Taltala High School and passed the Matriculation Examination of the University of Calcutta in 1950. In 1952 he passed the Intermediate-in-Science Examination of the University of Calcutta.

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"The Effect of Chemical Composition of Alkylbenzene Sulfonates on Adsorption by Soils," Master's Thesis, University of Illinois, Urbana, Illinois, June, 1963.

"Kinetics of Aerobic Utilization of Mixed Sugars by Heterogeneous Microbial Populations," Report WRC 0969, Water Resources Center, Georgia Institute of Technology, Atlanta, Georgia, 1969.

"Theoretical Aspects of Evaluating BOD Data Using Enzyme and Growth Kinetics," submitted for publication in Proceedings of the American Society of Civil Engineers.

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